QD
1
S923

ARKIV FÖR KEMI

UTGIVET AV

K. SVENSKA VETENSKAPSAKADEMIEN

Band 7

Med 64 avhandlingar

STOCKHOLM
ALMQVIST & WIKSELLS BOKTRYCKERI AB

LONDON
H. K. LEWIS & CO., LTD
136, GOWER STREET

1955

PARIS
LIBRAIRIE C. KLINCKSIECK
11 RUE DE LILLE

Häfte	1	innehållande	Nr 1—12	utkom	den	13 april	1954
»	2	»	Nr 13—20	»	»	14 april	1954
»	3	»	Nr 21—33	»	»	26 okt.	1954
»	4	»	Nr 34—41	»	»	28 okt.	1954
»	5	»	Nr 42—51	»	»	6 dec.	1954
»	6	»	Nr 52—64	»	»	21 febr.	1955

SJUNDE BANDETS INNEHÅLL

Sid.

1. Williams, R. J. P., Hagdahl, L., and Tiselius, A., Some further studies of chromatographic methods with particular reference to carbon adsorbents . . . 1— 15
2. Lindvall, S., and Carsjö, A., The influence of calcium on the relative distribution of phosphate compounds and ribonuclease between the particulate components and ground cytoplasm of the sea-urchin egg . . 17— 27
3. Brändström, A., Some remarks on the mechanism attributed to the reaction of 4-hydroxycoumarin with acetyl chloride . . . 29— 32
4. A.-Estrada, D., Kinetic study of the zirconium silicate / calcium oxide reaction . . . 33— 38
5. Pettersson, K., Optical resolution and configuration of 2-thienylsuccinic acid . . . 39— 48
6. Lindqvist, I., and Aronsson, B., The structure of the hexatantalate ion in $4\,K_2O \cdot 3\,Ta_2O_5 \cdot 16\,H_2O$ . . . 49— 52
7. Myrbäck, K., Starch degradation by salivary amylase II . . . 53— 59
8. Schotte, L., and Berglund-Larsson, U., An investigation of the mechanism for the formation of S-*tert*. alkyl thiuronium picrates . . . 61— 64
9. Brändström, A., The acidolysis of ethyl diacetoacetate. II. The effect of cobaltic tris-acetylacetonate . . . 65— 73
10. Åkerfeldt, S., On the isolation of ~~two~~ types of intermediates from the Strecker degradation . . . 75— 80
11. Brändström, A., Chelate alkali compounds in organic reactions. II. A kinetic study of the alkylation of different alkali compounds of ethyl acetoacetate . . . 81— 88
12. Brändström, A., The acidolysis of ethyl diacetoacetate. III. The importance of variation of the alcohol component of diacetoacetates in the magnesium oxide catalysed reaction with acetic acid . . . 89— 94
13. Brändström, A., The acidolysis of ethyl diacetoacetate. IV. Effect of variation of the alcohol component of alkyl diacetoacetates on the velocity of the reaction catalysed by sodium acetate . . . 95—101
14. Brändström, A., The acidolysis of ethyl diacetoacetate. V. Importance of coordination of the ester to the cation of the catalyst . . . 103—115
15. Wideqvist, S., The reaction between phenylsuccinyl chloride and ammonia . . . 117—120
16. Hasselqvist, H., Zur Kenntnis der Dehydro-Ascorbinsäure . . . 121—126
17. Lundblad, G., Proteolytic activity in sea urchin gametes. IV. Further investigations of the proteolytic enzymes of the egg . . . 127—157
18. Lundblad, G., Proteolytic activity in sea urchin gametes. V. The influence of ribonuclease upon the proteolytic activity of egg extracts 159—167
19. Lundblad, G., Proteolytic activity in sea urchin gametes. VI. A study of a proteolytic enzyme in extracts of spermatozoa . . . 169—180
20. Brändström, A., On the anionoid and cationoid substitutions of metallic derivatives of β-ketoesters and related compounds . . . 181—191

Sid.

21. FREDGA, A., and WESTMAN, L., Studies on synthetic growth substances. V. The configuration of the allyl-phenylacetic acid . . . . 193—195
22. SHERMAN, F. G., The uptake of Thorium B and Thorium C by yeast cells 197—204
23. THEORELL, H., and NYGAARD, A. P., The linkages between FMN and the protein of the "old yellow enzyme" studied by means of fluorescence measurements . . . . 205—209
24. LOURAU-PITRES, M., Incorporation du ^{14}C dans les graisses et le glycogène du foie chez des souris irradiées ayant reçu une injection de glucose marqué par du ^{14}C . . . . 211—223
25. HALVARSON, K., Some derivatives of 2-amino-4-ethylpyridine . . . . . 225—227
26. WIDEQVIST, S., Some aspects of Kolbe's synthesis of salicylic acid . . . 229—240
27. FREDGA, A., Studies on synthetic growth substances. VI. Optically active *p*-nitrohydratropic acid and *p*-aminohydratropic acid . . . . 241—247
28. SANDSTRÖM, J., On mercaptothiadiazines and *N*-aminothiazolethiones . 249—255
29. VON EULER, H., VON EULER, B., und HASSELQUIST, H., Untersuchungen am Yoshida-Ascites-Sarkom der Ratte . . . . 257—266
30. GRONOWITZ, S., On the preparation and Grignard reaction of 3-bromothiophene . . . . 267—271
31. LINDNER, R., und TEREM, H. N., Diffusion von radioaktivem Blei in Bleioxydationsschichten . . . . 273—277
32. PETTERSSON, K., The enanthiomorphs of α-(2-thienyl) hydrocinnamic acid and 2-thenylphenylacetic acid . . . . 279—285
33. MELANDER, L., Note on the kinetics of exchange of isotopes with appreciable isotope effect . . . . 287—289
34. MYRBÄCK, K., and WILLSTAEDT, E., Properties of Lintner starches . . . 291—314
35. HÖGFELDT, E., On the properties of binary mixtures. I. Influence of compound formation on activity factors and activities . . . . 315—337
36. PETTERSSON, K., Optical resolution of α-phenylhydrocinnamic acid and 2-thenyl-2-thienylacetic acid . . . . 339—346
37. PETTERSSON, K., Phase diagrams of phenylsuccinic and 2-thienylsuccinic acids. Configuration of phenylsuccinic acid . . . . 347—353
38. ÅKERFELDT, S., Studies on the spleen from rats fed with N,N-dimethyl-4-aminoazobenzene . . . . 355—360
39. GRONOWITZ, S., Metalation and halogen-metal interconversion in the thiophene series . . . . 361—369
40. HÄGG, G., and SCHÖNBERG, N., X-ray studies of sulfides of titanium, zirconium, niobium, and tantalum . . . . 371—380
41. WIDEQVIST, S., The association of racemic and optically active α-benzyl-α-cyanopropionic acid in benzene . . . . 381—386
42. FREDGA, A., and VÁZQUEZ DE CASTRO Y SARMIENTO, C., Studies on synthetic growth substances. VII. The enanthiomorphs of hydrocoumarilic acid . . . . 387—390
43. VON EULER, H., HASSELQUIST, H., und ERIKSSON, E., Einfluss von Tryptophan-Injektionen auf das rote Blutbild der Ratte . . . . 391—397
44. KIHLSTRÖM, J.-E., Incorporation of S^{35} in sperm-antagglutin . . . . . . 399—401
45. MYRBÄCK, K., and WILLSTAEDT, E., Starch degradation by salivary amylase. III. Residual dextrins and the glucose formation . . . . 403—415

Sid.

46. Edgar, R., und Norling, A., Elektronenmikroskopische und röntgenographische Studien an Superpolyamidfäden. Beziehungen zwischen dem Feinbau der Superpolyamidfäden und dem der Viscoseseide . . . . . . 417—426
47. Frick, G., Solubility differences of desoxyribonucleoprotein prepared from different sources . . . . . . 427—436
48. Matell, M., Stereochemical studies on plant growth regulators. IX. The enantiomorphic α-(4-chlorophenoxy)propionic acids and their configuration . . . . . . 437—442
49. Tiselius, A., Chromatography of proteins on calcium phosphate columns 443—449
50. Lundegårdh, H., Enzyme systems conducting the aerobic respiration of roots of wheat and rye . . . . . . 451—478
51. Schotte, L., and Rosenberg, A., Stereochemical studies on dicarboxylic acids I. The infrared absorption of some α, α′-dimethylsubstituted acids 479—491
52. Schotte, L., The diastereomers of 2,6-tetrahydrothiapyrandicarboxylic acid . . . . . . 493—500
53. Holmberg, B., Zur Kenntnis der Zuckerbenzhydrazide . . . . . . 501—512
54. Holmberg, B., Hexuronsäuren und Thiobenzhydrazid . . . . . . 513—515
55. Holmberg, B., Zur Kenntnis der Glykothiodiazoline . . . . . . 517—528
56. Holmberg, B., Über *l*-Fucose-thiobenzhydrazid . . . . . . 529—534
57. Porath, J., Charcoal chromatography with a step-graded adsorption column . . . . . . 535—537
58. Yamashina, I., Studies on enterokinase. I. The purification and general properties . . . . . . 539—543
59. von Euler, H., und Hasselquist, H., Untersuchungen am Yoshida-Ascites-Sarkom der Ratte. III . . . . . . 545—551
60. Jorpes, E., and Mutt, V., On the action of highly purified preparations of secretin and of pancreozymin . . . . . . 553—559
61. Högfeldt, E., On ion exchange equilibria. IV. The silver-hydrogen equilibrium on Wofatit K S at low silver concentrations . . . . . . 561—564
62. Lindner, R., und Spicar, E., Kontinuierliche Messung der Selbstdiffusion im Kalziumorthosilikat . . . . . . 565—575
63. Zdansky, G., The preparation of 2,4- and 3,4-dichlorohydratropic acids 577—581
64. von Euler, H., Hasselquist, H., und Glaser, A., Einfluss der Azidität auf Phenole, Cyclodiene, Endiole und Anile . . . . . . 583—587

ARKIV FÖR KEMI Band 7 nr 1

Read 14 October 1953

Some further studies of chromatographic methods with particular reference to carbon adsorbents

By R. J. P. Williams[1], L. Hagdahl and A. Tiselius

With 12 figures in the text

In a previous paper (1) a critical review of displacement analysis procedures has been undertaken. The paper presented and examined data on the adsorption of many substances onto carbon adsorbents and then described the effect of the presence of other strongly adsorbed substances upon this adsorption. In this paper further isotherm studies will be described for the adsorption of compounds onto carbon both from weak solutions of strongly adsorbed substances, which can act as saturators or displacing agents, and from more concentrated solutions of weakly adsorbed substances, eluting agents. The isotherms make it possible to analyse the effect of different developing agents on the movement of zones in chromatograms. Some theoretical comments on our findings are included in a second part of the paper.

The determination of the adsorption of a substance at a given concentration was carried out in the manner described earlier (1), i.e. by the method of frontal analysis. As a general inspection of the isotherms of a large number of compounds in different concentrations of many second components was required it was decided to determine the adsorption only in concentration regions in which substances are applied to columns in normal chromatographic procedures. For a rough description of most of the isotherms, then, only two concentration points were obtained—at 0.1 % and 0.5 % weight/volume of solvent. The isotherm points so obtained do not allow an exact description of the isotherm, of course, but they do provide information which gives a rough picture of the extent of adsorption and its variation with concentration. Before proceeding to a theoretical account of the controlling factors in the mathematical equations corresponding to these isotherms, we will give the experimental data which have been recently obtained.

Part I. Isotherms

Experimental data

The first set of isotherms illustrating the effect of different eluting agent concentrations upon adsorption are given in Fig. 1. They are those of the saccharide, raffinose, in the presence of different concentrations of ethanol, the adsorbent being Darco G 60 carbon. A similar set of isotherms have also been obtained on

---

[1] Present address: Merton College, Oxford, England.

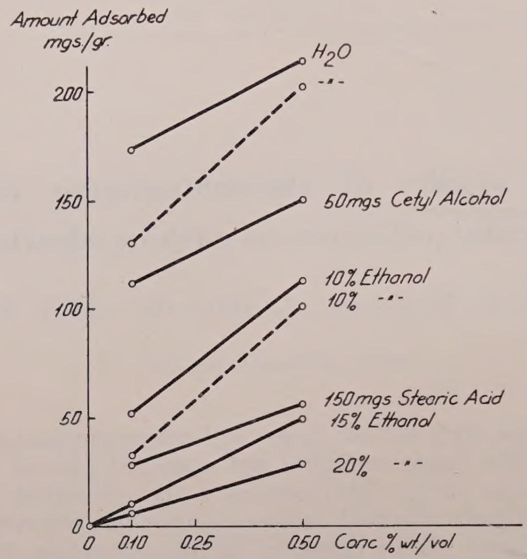


Fig. 1. Adsorption isotherms of raffinose onto Darco G 60 carbon in the presence of different ethanol/water solvents *or* after the specified pretreatment of the adsorbent, by cetyl alcohol or stearic acid, given as mgs/g carbon. The broken lines are isotherms on Carboraffin Supra charcoal.

other carbons, e.g. Carboraffins, Fig. 1. From the figure it is clear that the isotherms in water are very strongly curved even at low concentrations, where the adsorption is very strong. It is to be anticipated therefore that the elution of raffinose from carbon adsorbents by water will result in an unsymmetrical elution peak with considerable "tailing" and that the desorption will tend to be slow. Both of these properties can be observed experimentally, and hence normal elution procedures with water are of little value in the isolation of raffinose (2).

From the nature of the adsorption isotherm of phenylalanine onto Darco G 60 carbon from water, Fig. 2, the same deductions can be drawn as in the case of raffinose. Experimentally it is readily demonstrated that phenylalanine cannot be satisfactorily eluted from carbon with water. Other chromatograms have shown that this is also true of many amino acids and saccharides.

Figs. 1 and 2 illustrate two points. (a) The greater the concentration of the eluting agent the closer does the isotherm approach linearity up to concentrations of 0.5% of the compound adsorbed. (b) The greater the concentration of the eluting agent the smaller the capacity of the adsorbent at a given concentration of the substance adsorbed.

Both saccharides and amino acids are more soluble in water than in ethanol solution. As it was considered that solubility might be affecting the changes in adsorption with changes of solvent a number of isotherms were obtained for the adsorption of fatty acids and of fatty alcohols in the same solvents. These substances are less soluble in water than in ethanol. Fig. 3 taken from a paper by Hagdahl and Holman (3), gives a number of the isotherms of the fatty acid, caprylic acid, in different ethanol solutions. The next figure, fig. 4, gives similar information for

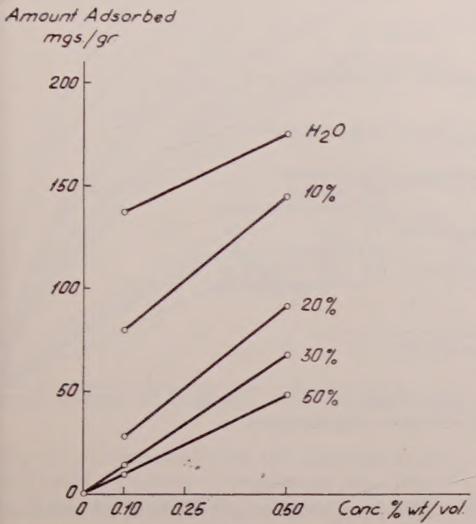


Fig. 2. Adsorption isotherms of phenylalanine onto Darco G 60 carbon in the presence of different ethanol/water solvents.

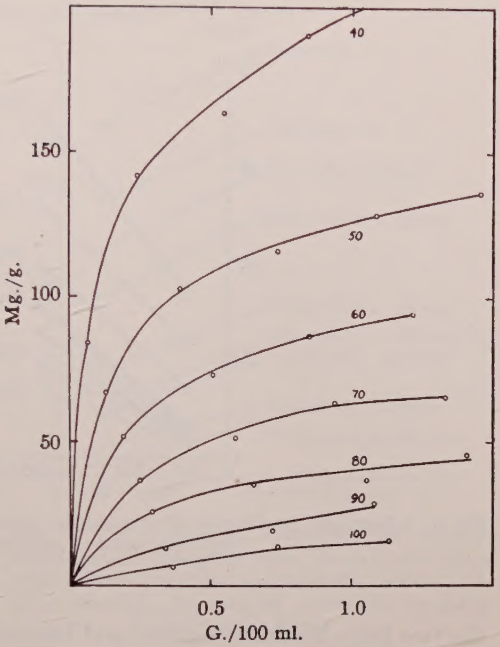


Fig. 3. Adsorption isotherms of caprylic acid onto Darco G 60 carbon in the presence of different ethanol/water solvents. The numbers in the diagram represent % wt./vol. ethanol.

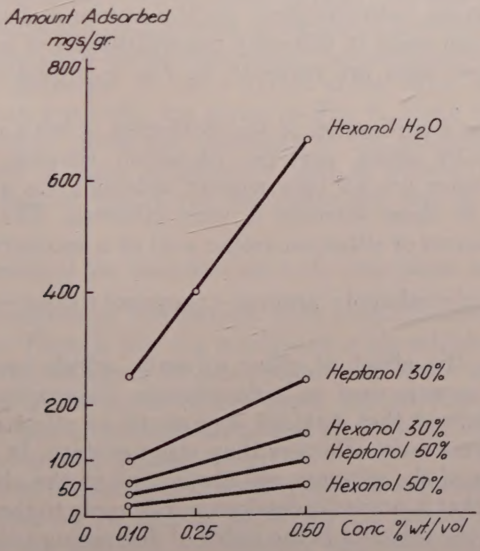


Fig. 4. Adsorption isotherms of two alcohols, hexanol and heptanol, onto Darco G 60 carbon from water/ethanol solvents. The percentages are of ethanol.

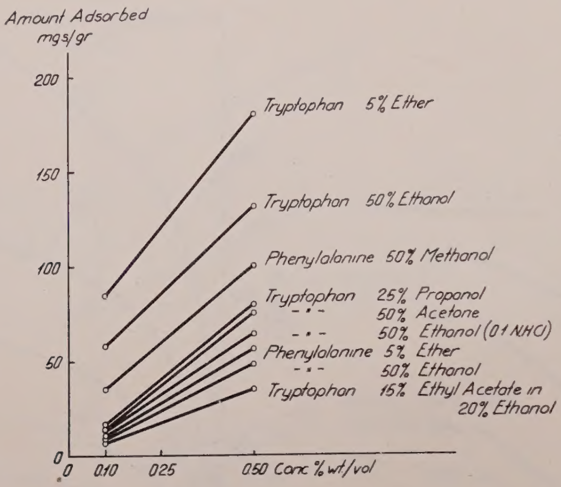


Fig. 5. Adsorption isotherms of tryptophan and phenylalanine onto Darco G 60 carbon from different water/,,second solvent" mixtures.

the two fatty alcohols, hexanol and heptanol. The appreciable adsorption of hexanol and heptanol as well as of the higher fatty acids in ethanol-containing media makes these substances suitable as saturators, when ethanol-water is used as an eluting agent (4), (5), and (6).

All the isotherms so far given here illustrated effects apparent in ethanol/water systems but many alternative combinations of eluting agents exist. In order to understand the differences between these eluting agents a study was made of the isotherms of some amino acids in different concentrations of a number of solvents in water. Some of these data are recorded in Fig. 5, which reveals a number of striking features.

In the first place the modification of the isotherms of both phenylalanine and of tryptophan produced by ether, acetone, propanol, ethanol, ethyl acetate, and acetone solutions in water are all very similar although the quantitative effect of equal concentrations of these solvents is very different. The order of increasing depression of the isotherms of either an amino acid or a saccharide would seem to be

$$\text{water} < \text{methanol} < \text{ethanol} < \text{acetone} < \text{propanol} < \text{ether} < \text{ethylacetate}.$$

In order to ascertain the effect of other solvents, which are insoluble in water, ethanol/water mixtures were used as basic solvents. Comparison with the members of the above series showed that butanol was about as effective as ether and that benzene and hexane were more effective than ethyl acetate. It is not to be expected that the eluting power of the solvents will always fall in the above series but recent experiments indicate that a similar order does in fact apply to the elution of fatty acids.

The above order of the solvents is the order of decreasing polarity or of increasing chain length of the members of an homologous series. This is exactly the opposite of the order obtained for the effective eluting power of the various solvents upon

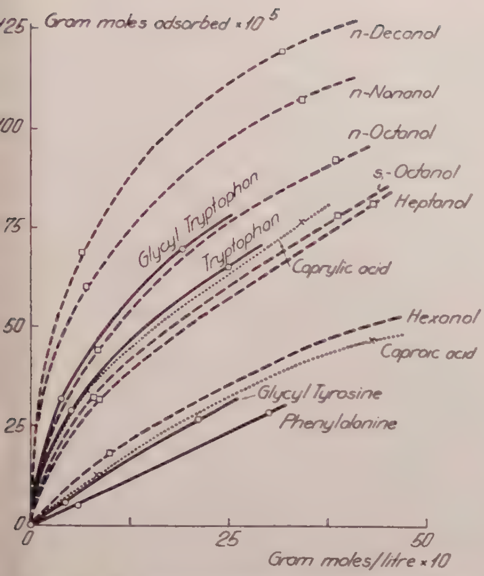


Fig. 6. A comparison of the adsorption isotherms of a number of substances onto Darco G 60 carbon from 50 % ethanol/water.

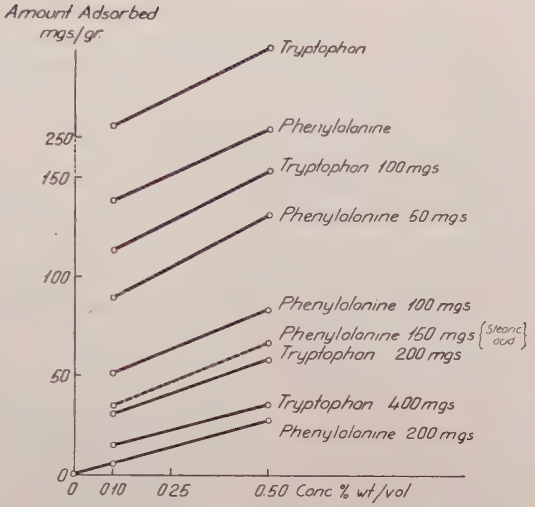


Fig. 7. The adsorption isotherms of phenylalanine and tryptophan onto Darco G 60 carbon after different pretreatments of the carbon with cetyl alcohol. Amount of saturator recorded in mgs/g carbon.

substances adsorbed onto a hydrophilic adsorbent such as silica or alumina. Thus chromatography on carbon should be complementary to that on silica or alumina.

Before leaving the question of the effect of eluting agents upon adsorption isotherms a further point arises. In a previous paper (1) it was pointed out that if the isotherms for a substance and its displacer, or carrier in a carrier displacement analysis, did not cross then there was a better chance that the conditions of "ideal" displacement might operate. The isotherms of some amino acids, fatty acids, and alcohols in 50 % ethanol/water as a solvent are given in Fig. 6. None of the isotherms cross, and neither would they cross the isotherms of the saccharides. Displacement in such a mixed solvent is more satisfactory than in water (7) (8). Carrier displacement may also be carried out if the isotherms are not to close.

The effect of pretreatment of the adsorbent upon the adsorption isotherms.

So far only modifications in the solvent have been considered in the attempt to adjust the adsorption. There is also the possibility of the adjustment of the adsorbent to be considered. Some isotherms for adsorption from water onto pretreated carbon adsorbents have been given in an earlier paper (1). In Figs. 7 and 8 further isotherms for the adsorption of phenylalanine and tryptophan onto pretreated carbons (Darco G 60 and Carboraffin Supra) are given.

From Figs. 7 and 5 it is seen that the adsorption is depressed in a different manner by a saturator than by an eluting agent. In the former case different degrees of saturation depress the isotherm increasingly and seemingly in such a way that the higher concentration regions of the isotherms remain parallel, or very nearly so, with the original isotherm in the absence of any saturation. This would suggest

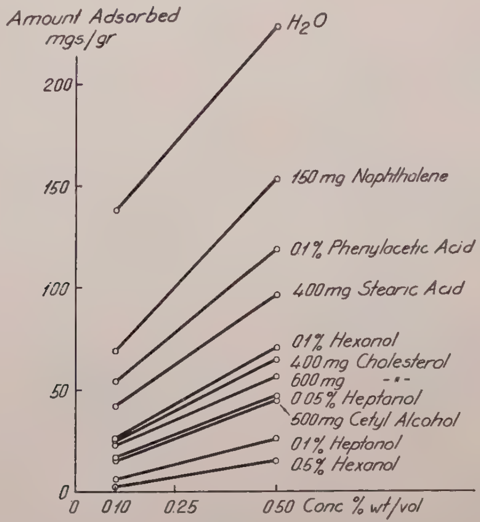


Fig. 8. The effect of different saturators, applied as in refererne (1) pages 206 and 216, on the adsorption isotherm of phenylalanine onto Carboraffin Supra carbon from water.

that certain sites are eliminated by the saturator progressively and that these sites are those that account for the very strong adsorption at low concentration where the adsorption rises very rapidly with concentration. Another fact of interest from Figs. 7 and 8 is that there are no specific features of the pretreatment. This is peculiar in view of the use of very different compounds as saturators for it might well have been thought that a compound such as stearic acid with its highly polar acid group might have acted as an ion exchange site whereas no such property would have been expected when pretreatment was carried out with cetyl alcohol. The same points can be made by an analysis of the isotherm data for the saccharide raffinose which is presented in fig. 1. On page 12 a more theoretical approach is made to these data.

There is a second way in which specificity of a saturator can appear. A given compound when it is adsorbed onto carbon is adsorbed on certain sites but it is by no means certain that all substances will occupy the same sites as the surface of a carbon adsorbent must include a wide diversity of "active spots" and pore sizes. If this is to be expected then it would also follow that a saturator would depress the adsorption of a given compound in a specific manner. Thus it may be found that two molecules which are very similar in chemical and physical properties will affect the adsorption of one another much more than two dissimilar compounds. This point has already been mentioned in the paper on displacement (1) where it was observed that "ideal" displacement (an effect shown to be similar to efficient saturation) was obtained most clearly in homologous series and in series of like compounds such as amongst the amino acids. These observations have been confirmed for saturators.

A comparison of the number of molecules of a saturator applied to the adsorbent and the reduction in adsorption (in numbers of gram molecules) of given compounds by this saturation reveals that there is no simple relationship between these two quantities. Thus cetyl alcohol as a saturator would appear to be able to reduce the adsorption of tryptophan more effectively than that of phenylalanine, and in turn even more effectively than that of raffinose. One molecule of this saturator, on the average, prevents the adsorption of more than two molecules of tryptophan and less than one molecule of raffinose. It seems that the saturator reduces the adsorption of the most strongly adsorbed substances by the greatest amount which will, of course, reduce the separating efficiency of the adsorbent. (For definition of separating efficiency see page 10 below.) There are very few data on the effect of different saturators. Stearic acid is almost exactly as effective as cetyl alcohol in preventing the adsorption of both amino acids and saccharides. Equal amounts, as gram moles, of other saturators appear to be either more or less effective according to whether they are more or less strongly adsorbed. Thus very large amounts of naphthalene are far less effective than much smaller amounts of zephiran chloride.[1]

Again an almost linear relationship exists between the quantity of a given saturator employed and the reduction in adsorption which it produces, Fig. 9. Different saturators, or the same saturator used to prevent the adsorption of other compounds, would give a line of very different slope in the figure. There is a definite limit to the linear effect of saturation which becomes almost independent of the amount of the saturator above a certain value.

The adsorption of a given substance onto different charcoals follows markedly different isotherms. This is illustrated in Figs. 10 and 11 for the adsorption of phenylalanine and hexanol. A similar point was made by HAGDAHL and HOLMAN in a discussion of the adsorption isotherms of fatty acids on different charcoals (3). Although there are definite differences between the different carbons these differences are similar for phenylalanine as for fatty acids. Despite this evidence ALM (5) has shown recently that in the gradient elution analysis of polysaccharides different carbons exhibit specific adsorption differences.

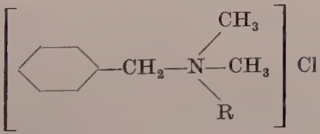
The next part of this paper will discuss the significance of these isotherm studies for a critical analysis of the separating efficiency of column procedures.

Part II. The separating efficiency of chromatographic procedures

In this part of our paper an attempt will be made to express the implication of the isotherm data, collected in this and a previous paper (1) in chromatographic studies. The above experiments were designed to find methods of adjusting where possible the adsorption isotherms of compounds, in order to make the adsorption characteristics such that the substances could be developed as well defined zones in usual chromatographic procedures, e.g. in elution or displacement analysis. Two main

---

[1] Zephiran chloride is the trade name for a detergent consisting of a mixture of compounds of general formula



where R are alkyl radicals, C_8H_{17}—$C_{18}H_{37}$.

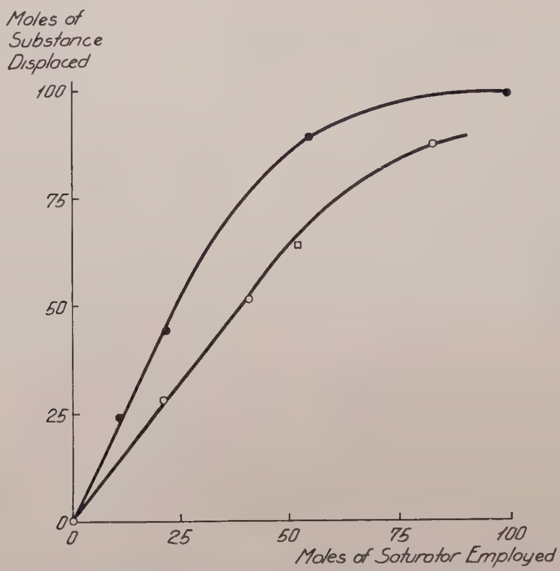


Fig. 9. The depression of adsorption of phenylalanine by saturation of Darco G 60 carbon. Saturation with cetyl alcohol, ○; stearic acid, □; and cholesterol stearate, ●.

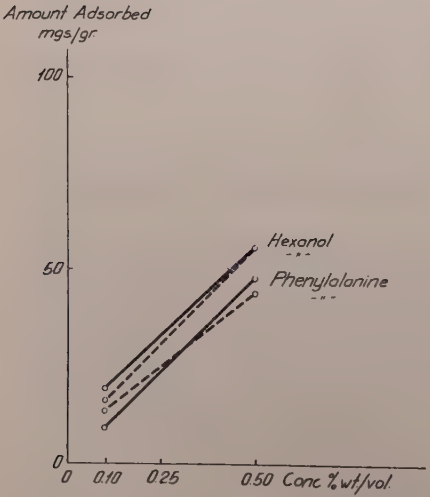


Fig. 10. Adsorption isotherms of hexanol and phenylalanine onto two carbons from 50 % ethanol. Full lines—Darco G 60, broken lines—Carboraffin Supra.

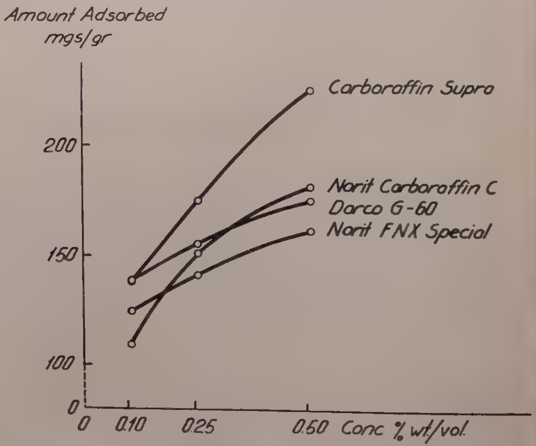


Fig 11. Adsorption isotherms of phenylalanine onto different carbons from water, N.B.: The ordinate axis commences at 100 mgs/g.

ways of adjusting the isotherms have been noted; firstly that brought about by a high concentration of a weakly adsorbed substance, an eluting agent, and secondly that brought about by a low concentration of a strongly adsorbed substance, a displacer or a saturator. At the same time it was realised that intermediate changes would be brought about by an intermediate class of substances "non-ideal" displacers (1). The introduction of eluting or displacing agents does not only modify the mathematical form of the isotherm but their presence also alters the capacity and separating efficiency of unit quantity of the adsorbent in a chromatographic column. A satisfactory adjustment of the isothermal adsorption must produce a desirable relationship between the residual adsorption and the concentration of the material in the solution (see below), must eliminate irreversible adsorption, and simultaneously maintain a high capacity and separating efficiency in the system. A theoretical approach to such a problem will be attempted.

The linear isotherm

The simplest relationship between the amount of the substance adsorbed, m, and the concentration of it in solution, c, is that of direct proportionality found in the linear isotherm, which closely resembles the Nernst partition law.

$$m = k \cdot c. \tag{1}$$

For the chromatographic development of substances following such an isotherm simple elution with a single solvent can be employed to advantage because the rate of movement of a zone of such a substance in elution is independent of the concentration of the substance. MAYER and TOMPKINS (10) following the treatment of MARTIN and SYNGE (11) for the case of partition chromatograms, have derived an expression for the separating efficiency of such a procedure when equation (1) is obeyed. They showed that complete separation was never possible, except at infinite column length, due to the theoretical spreading involved in simple transfer operations but the degree of separation that could be achieved in unit amount of the column was found to be proportional to the individual values of k in equation (1). Although displacement must play some part in the initial stages of the separation, the experiments of MAYER and TOMPKINS showed that the effect must be very small in their systems, as the experimental elution peaks had a theoretical shape even in the region where two zones overlapped. SPEDDING (12) using higher concentrations, however, obtained results illustrating such displacements.[1]

---

[1] The close fit between the experimental and the theoretical elution peaks in these experiments is not easy to understand. In the work of MAYER and TOMPKINS ion exchange resins are used as adsorbents for trivalent rare earth ions. The activity of these ions both in the liquid and in the resin is not linearly proportional to the concentration. Thus the amount of such an ion adsorbed is unlikely to be directly proportional to the concentration of the ion in solution. MAYER and TOMPKINS (10) however assume that just such a relationship holds. It seems that at the very low concentrations which they employ the conditions are very different from those obtaining in the experiments of SPEDDING (12) who uses displacement analysis on similar exchange resins. A large number of equilibrium adsorption studies on exchange resins would also suggest that the simple mass action law, which must hold if a linear isotherm is to obtain at low concentrations, is not obeyed (13).

The Langmuir isotherm

This isotherm, equation (2) is not linear

$$m = \frac{a \cdot b \cdot c}{1 + b \cdot c} \tag{2}$$

where m is the amount of the substance adsorbed, at concentration c, and a and b are constants. Substances obeying this adsorption isotherm can be developed by an elution procedure in a chromatographic analysis but the effluent peaks obtained are unsymmetrical due to the different rate of travelling of different concentration regions. Thus such substances are usually developed by displacement. However it is the mutual displacement of substances during elution which is of most interest here. GLUECKAUF (14) has described the process in detail and has shown that the separating efficiency of the adsorbent can be expressed in terms of the column length X_0 required to obtain *complete* separation. The separating efficiency, S, will clearly be inversely proportional to this quantity.

$$S = \frac{1}{X_0} = \frac{\left(\frac{b_2}{b_1} - 1\right)^2 \cdot a}{m_1 + m_2} \tag{3}$$

where the symbols have the same meaning as in equation (2) and the sub-scripts refer to two different substances. It is worth noting that the equation applies to the adsorption of two compounds which cannot be adsorbed onto different sites, and that no further separation can be achieved by utilising a longer column than X_0. Again equation (3) does not apply to the normal displacement method in which both components under investigation are displaced by a third, the developer. This latter technique brings about more efficient separation (14) but the final zones move adjacent to one another.

Changes in a and b in equation (2) modify the isotherm in rather different ways, Fig. 12. This figure can be compared with Figs. 5 and 7, when it will be seen that the change of the b constants in the Langmuir isotherm parallels the changes produced by an eluting agent whereas the change in the value of a in the same equation parallels the changes produced by a saturator. The constant b in the Langmuir equation can be related to the affinity of the adsorbent for the substance and the constant a with the number of available sites for adsorption. This leads to the further proposition that saturators and ideal displacers act by blocking adsorbent sites but that eluting agents chiefly act by reducing their affinity without blocking them.

The effect of changes of the constants in the Langmuir equation is of interest in equation (3). In the first place the separating efficiency decreases linearly with decreasing a. Secondly it is not directly dependent upon the value of b but only on the ratio $\frac{b_2}{b_1}$. If this ratio is kept constant during the adjustment of the isotherm whilst large changes are made in the b values, other things being constant, there is no loss of efficiency. Both a reduction in b and a change of the load to smaller values,

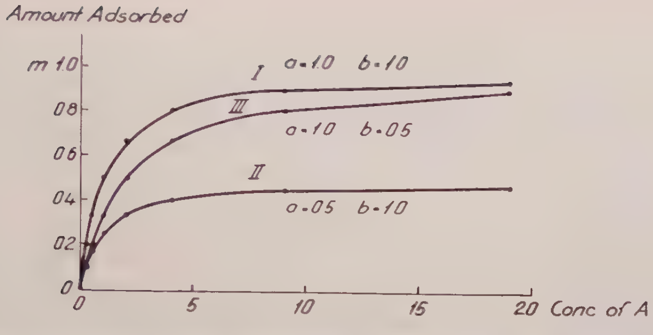


Fig. 12. The effect of changes in the coefficients a and b of the Langmuir isotherm upon the variation of adsorption with concentration.

which implies the use of lower concentrations of the substances, are desirable, for the Langmuir equation (2) then approaches more closely to the linear equation (1) and hence the spreading in elution development due to the different rates of travelling of regions of different concentration, is eliminated.

The Freundlich isotherm

Generally speaking the adsorption of a substance from a liquid phase onto a solid does not follow either of the above equations (1) and (2) but tends rather to the empirical form

$$m = p \cdot c^{\frac{1}{n}} \tag{4}$$

where m and c have their usual meaning and n and p are constants. Elution or displacement analysis can be shown to be mostly unsatisfactory in the case of substance for which adsorption follows equation (4), the Freundlich isotherm. The rate of travel of an infinitely small concentration of such substances down a chromatographic column is infinitely slow so that zones of the substances in elution tend to tail back to the top of the column. Furthermore GLUECKAUF (16) has shown theoretically and we have shown experimentally (1) that displacement is more likely to be a failure than a success for many pairs of these substances. The modification of the Freundlich isotherm to a simpler form would seem to be demanded if the adsorbent/substance systems for which the Freundlich isotherm is obeyed are to be utilised. As almost all the adsorption onto carbon which we have studied seems to fit the Freundlich isotherm more closely than either the linear or the Langmuir isotherms, we decided to investigate how adsorption following the Freundlich isotherm could be modified. The experimental isotherms have all been described above and in (1) but the mathematical form of these isotherms has not been discussed. By plotting the logarithm of the concentration % by weight of the substance against the logarithm of the amount of it adsorbed, mgs/g carbon, we obtained the values of the Freundlich coefficients given in Table I. Some data from another paper (15) are also given in the table. Before discussing the results in terms of column efficiency there are a few points of general interest.

(a) The isotherm coefficients $\frac{1}{n}$ for adsorption from water of members of different homologous series are more different, in general, than are the differences between members of the same homologous series for similar log p values.

This can be expressed by saying that the isotherms of members of different homologous series tend to cross one another whereas isotherms of members of the same homologous series do not (1). The values of $\frac{1}{n}$[1] found, also lead, utilising the argument of GLUECKAUF (7), to the prediction that, as these values are larger for the alcohols than for the amino acids and sugars (generally speaking), there would be considerable difficulty in displacing an amino acid or a saccharide with an alcohol especially at low concentrations. Now the experimental facts about such displacements have been presented (1), and it was found that the alcohols did not act as ideal displacers for the amino acids in many cases even when the isotherm of the alcohol appeared to lie entirely above that of the amino acid. At larger concentrations however displaced zones could be obtained with step-heights similar to those predicted from the simple diagrammatic outline of displacement given by Tiselius (compare ref. (1)). However, if members of the same homologous series were used to displace one another then it was observed that displacement was nearly ideal (1), the more strongly adsorbed member travelling as the rear zone, despite the isotherms of the substances obeying the Freundlich equation. These observations are consistent with the theoretical arguments of GLUECKAUF (17) in that $\frac{1}{n}$[1] is generally in fact the larger the lower the adsorption of the member of the homologous series.

(b) Eluting agents affect the value of $\frac{1}{n}$ more than they affect the values of log p in the isotherms of the amino acids and saccharides and in many cases the isotherm finally reverts to a linear isotherm $\left(\frac{1}{n} = 1.0\right)$ in high eluting agent concentrations. The same effect is not present so markedly in the case of hexanol (cf. phenylalanine) for which substance the isotherm coefficient log p is more markedly influenced by the eluting agent than is $\frac{1}{n}$. This may well reflect a reduction in cooperative adsorption upon the change of solvent from water to ethanol (2). (c) The effect of a saturator is also different for different compounds. In the case of saturation with 0.1 % hexanol for example the isotherm coefficient which is most affected for amino acids and saccharides is log p, whereas $\frac{1}{n}$ is but little adjusted until very high saturations and until log p has become very small. In the case of adsorption of the fatty alcohols there is very little change in the values of either log p or of $\frac{1}{n}$ after saturation and it would seem that the alcohols are soluble in the saturator — a partition system being set up (1).

---

[1] It should be noted that the value of n used by GLUECKAUF is not the same as that used here as GLUECKAUF uses another form of the Freundlich isotherm (17).

Table I. The coefficients in the Freundlich isotherm.

Substance	Solvent	Adsorbent	log p	$\frac{1}{n}$	Lit. ref.
Glycyl tryptophan	Water	Carboraffin Supra	0.85	0.32	(1)
Trytophan	»	»	0.61	0.26	»
Glycyl tyrosine	»	»	0.57	0.30	»
Phenylalanine	»	»	0.42	0.32	»
Leucyl glycyl glycine	»	»	0.33	0.40	»
Leucine	»	»	−0.20	0.55	»
N-hexanol	»	»	0.98	0.59	»
N-pentanol	»	»	0.58	0.50	»
Iso-pentanol	»	»	0.50	0.51	»
N-butanol	»	»	0.30	0.60	»
N-propanol	»	»	−0.03	0.67	»
Tryptophan	Water with 0.1 % hexanol as a saturator	»	0.24	0.37	»
Glycyl tryptophan	»	»	0.16	0.47	»
Phenylalanine	»	»	0.00	0.53	»
Leucyl glycyl glycine	»	»	−0.22	0.80	»
N-pentanol	»	»	0.51	0.95	»
Iso-pentanol	»	»	0.35	0.90	»
N-butanol	»	»	0.00	0.92	»
Acetic acid	Water	Darco G 60	−0.87	0.49	(15)
Propionic acid	»	»	−0.63	0.42	»
Butyric acid	»	»	−0.42	0.34	»
Phenylalanine	»	»	0.29	0.17	Fig. 2[1]
»	10 % ethanol	»	0.27	0.37	»
»	20 % »	»	0.19	0.74	»
»	30 % »	»	0.13	1.00	»
»	50 % »	»	0.00	1.00	»
»	50 % methanol	»	0.20	0.65	Fig. 5
»	5 % ether	»	0.10	1.00	»
Tryptophan	Water	»	0.56	0.16	Fig. 7
»	50 % ethanol	»	0.32	0.56	Fig. 5
»	5 % ether	»	0.42	0.50	»
»	25 % propanol	»	0.18	0.96	»
»	50 % acetone	»	0.14	1.00	»
Hexanol	Water	»	1.00	0.66	Fig. 4
»	30 % ethanol	»	0.55	0.60	»
»	50 % »	»	0.00	1.00	»
Phenylalanine	Water	»	0.29	0.17	Fig. 7
»	»	» + 50 mg/g cetyl alc.	0.17	0.17	»
»	»	» + 100 mg/g » »	0.02	0.30	»
»	»	» + 150 mg/g stearic acid	−0.08	0.35	»
»	»	» + 200 mg/g cetyl alc.	−0.55	1.00	»
Tryptophan	Water	»	0.56	0.16	»
»	»	» + 100 mg/g » »	0.24	0.16	»
»	»	» + 200 mg/g » »	−0.10	0.37	»
»	»	» + 400 mg/g » »	−0.30	0.60	»
Raffinose	Water	Darco G 60	0.39	0.17	Fig. 1
»	10 % ethanol	»	0.24	0.54	»
»	15 % »	»	−0.02	1.00	»
»	20 % »	»	−0.30	1.00	»
»	Water	»	0.39	0.17	»
»	»	» + 50 mg/g cetyl alc.	0.23	0.19	»
»	»	» + 150 mg/g stearic acid	−0.15	0.37	»
»	»	Carboraffin	0.45	0.39	(2)
Maltose	»	»	0.31	0.55	»
Saccharose	»	»	0.26	0.37	»

---

[1] The figure numbers refer to the present paper.

In order to see what factors may be varied in the hope of adjusting the Freundlich isotherm into a more suitable form, the empirical equation (4) cannot be used. However if the Freundlich equation is re-expressed in terms of a sum of Langmuir terms, as suggested by HINSHELWOOD, then the constants in the equation have a physical meaning, see page 10.

$$m = \sum \frac{a \cdot b \cdot c}{1 + b \cdot c}. \tag{5}$$

This equation can be treated in the same manner as the discussion of the simple Langmuir equation (2) for it represents the summation of adsorption upon a series of Langmuir sites. The effect of a saturator will be to remove some of these sites whilst the effect of an eluting agent will be to reduce the affinity of the sites for the substance but the sites will not be blocked. However it is clearly very difficult to use a saturator in order to modify the isotherm for both the earlier theoretical ideas and the experimental evidence in Table I show that saturation does not readily lead to a linear isotherm. The only use for a saturator would appear to be in the elimination of certain unwanted sites. The effect of an eluting agent however will be to adjust the isotherm until it reaches a linear form as shown by the earlier arguments and by the data in the table. Furthermore it is easy to see that, once again, the use of an eluting agent as a developer may not greatly reduce either the capacity or the separating efficiency. In fact however it is only the most weakly adsorbed substances which can be satisfactorily eluted even in strong eluting agents if their adsorption in water follows a Freundlich isotherm (6). For the more strongly adsorbed substances it is essential to use both strong saturation to greatly reduce the adsorption and to eliminate the irreversibility, and to develop the chromatogram with rather powerful eluting agents. In this way although some of the separating power of the adsorbent is lost many satisfactory analyses have been achieved, on carbon adsorbents (4), (5), (6).

From the foregoing discussion the parallel changes in the form of the Langmuir equation upon the change in the constants, a and b, Fig. 1, with the experimental changes in the adsorption isotherms of substances obeying the Freundlich isotherm, see Figs. 7 and 5, can be understood. The similarity is a reflection of the similarity between equation (2) and equation (5).

Finally we would point out that the above considerations led us to consider the advantages of gradient elution procedures (4). In particular we have shown that even when adsorption follows the Freundlich isotherm, as in such cases as the adsorption of the amino acids, Fig. 11, and saccharides, Fig. 12, on carbon, substances can be eluted as discrete zones using this method (5), (6). Additional evidence is provided by the recent, and independent, examination of fatty acids by gradient elution from silica (18) and by the fractionation of high polymers to be reported in detail shortly. In many of these elution analyses the adsorbent was pretreated in order to block irreversible adsorption.

This work has been financially supported by the Swedish Natural Science Research Council. One of us (R. J. P. W.) would like to express his thanks to Rotary International for a Fellowship which has enabled him to take part in this work.

Institute of Biochemistry, University of Uppsala, Sweden.

REFERENCES

(1). L. Hagdahl, R. J. P. Williams, and A. Tiselius. Arkiv för Kemi *4*, No. 10, 193, 1952.
(2). A. Tiselius and L. Hahn. Kolloid-Zeitschrift *105*, 3, 1943.
(3). L. Hagdahl and R. T. Holman. J. Amer. Chem. Soc. *72*, 701, 1950.
(4). R. S. Alm, R. J. P. Williams, and A. Tiselius. Acta Chem. Scand. *6*, 826, 1952.
(5). R. S. Alm. Acta Chem. Scand. *6*, 1186, 1952.
(6). R. J. P. Williams. The Analyst. 77, 905, 1952.
(7). C. H. Li, A. Tiselius, K. O. Pedersen, L. Hagdahl, and H. Carstensen. J. Biol. Chem. *190*, 317, 1950.
(8). J. Porath. Acta Chem. Scand *6*, 1236, 1952.
(9). R. L. M. Synge and A. Tiselius. Acta Chem. Scand. *3*, 321, 1949.
(10). S. W. Mayer and E. R. Tompkins. J. Amer. Chem. Soc. *69*, 2866, 1947.
(11). A. J. P. Martin and R. L. M. Synge. Biochem. J. *35*, 1358, 1941.
(12). F. H. Spedding. Discussions of the Faraday Soc. No. 7, 214, 1949.
(13). J. F. Duncan and B. A. J. Lister. Quarterly Reviews. Chem. Soc., London, *2*, 307, 1948.
(14). E. Glueckauf. Proc. Royal Soc. *186 A*, 35, 1946.
Idem. Discussions of the Faraday Soc. No. 7, 12, 1949.
(15). F. H. Max Nestler and H. G. Cassidy. J. Amer. Chem. Soc. *72*, 680, 1950.
(16). E. Glueckauf and J. I. Coates. J. Chem. Soc. 1302 and 1315, 1947.
(17). E. Glueckauf. J. Chem. Soc. 1308 and 1321, 1947.
(18). L. M. Marshall, K. O. Donaldson, and F. Friedberg. Analytical Chemistry *24*, 773, 1952.

Tryckt den 4 mars 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 2

Communicated 11 November 1953 by JOHN RUNNSTRÖM and GEORGE DE HEVESY

The influence of calcium on the relative distribution of phosphate compounds and ribonuclease between the particulate components and ground cytoplasm of the sea-urchin egg

By SVEN LINDVALL and ASTRID CARSJÖ

With 5 figures in the text

In experiments with the so-called surface precipitation reaction in sea-urchin eggs, HEILBRUNN (5, 6) has shown that calcium ions are the only ions present in sea-water with the power of evoking this phenomenon. He has also demonstrated that the reaction is associated with certain degradation of the cytoplasmic granules. A similar degradation of particulate fractions has been established by HULTIN (8, 9) after treatment of homogenates of unfertilized eggs with calcium. In these experiments he showed that the addition of calcium gave rise to an increase in the oxygen uptake and to a formation of acid. He also found an increase in the viscosity, and especially in that of the concentrated homogenates. The author considered this viscosity effect to be due to a sulphhydryl mechanism, although he did not exclude the possibility of a coagulation system activated by calcium. The material liberated in the breakdown of the granules was also believed to contribute to the increase in viscosity.

The fertilization of the sea-urchin egg is also attended by an increase in viscosity, as has been shown by HEILBRUNN (5, 6) and by RUNNSTRÖM (23), so that the phenomena recorded by HULTIN may perhaps be compared with the process in vivo. MIRSKY (19) applied more quantitative methods and showed, using frozen-dried material, that the solubility of the proteins was altered in connection with the fertilization. In further work on this change in solubility, however, LINDVALL and CARSJÖ (16) have demonstrated that the changes observed in frozen-dried eggs appear during the freezing and drying procedures. The fraction which undergoes the change in solubility consists, apparently, of a nucleoprotein. In this connection the ribonuclease discovered by BERNSTEIN (2), which occurs in the unfertilized egg and whose activity decreases after the fertilization, may well be of interest.

On treatment of unfertilized eggs with ATP, RUNNSTRÖM and KRISZAT (24), using the centrifuge microscope, found that the rigidity of the cytoplasm increased. They concluded that the treatment conferred upon the cytoplasm a structure resembling that in the fertilized egg. ATP clearly favours a gel formation or other polymerization in the cytoplasm: according to LAKI and CLARK (13) and to SPICER (27), ATP causes gel formation in actomyosin. By extracting fertilized eggs, labelled with radioactive phosphate, first with ammonium sulphate solution and then with trichloroacetic acid, LINDBERG (15) has shown that the orthophosphate and ATP fractions are not homogeneous in the egg, but possess a certain heterogeneity, which is presumably of a cytochemical nature.

In the present investigation a study was made of the effects of calcium ions on different fractions of the egg. The phosphate-rich components and the ribonuclease were subjected to more detailed experiment.

Experimental

1. Effect of Ca ions on the homogenization

Unfertilized eggs of *Paracentrotus lividus* were employed. After repeated washings with filtered sea-water, the eggs were freed from their jelly coats with sea-water containing HCl. They were then washed three times with 3.8 % NaCl solution to remove calcium. The homogenization was effected by repeated passages of an egg suspension (1 ml eggs + 7 ml 3.8 % NaCl solution) into and out of a pipette with a narrow opening, until microscopical examination showed that no intact eggs were present. The homogenate was divided into a particulate fraction and a supernatant by centrifugation in an Ecco high-speed centrifuge at 8000 g for 30 min. The supernatant was fractionated further by addition of trichloroacetic acid to a final concentration of 12.5 %. The high-molecular components were thus precipitated and were centrifuged off, so that a clear solution of the low-molecular compounds in the supernatant was obtained.

The homogenate, the supernatant and the TCA-soluble fraction of the latter were analysed for phosphorus and nitrogen.[1]

For the phosphorus estimations the material was heated with 0.3 ml perchloric acid in pyrex tubes immersed in a salt bath at 160–180° C. After the combustion the determination was continued by the procedure of ALLEN (1). The sample was diluted to 10 ml, after which 0.2 ml 10 % ammonium molybdate solution and 1 ml of a 1 % solution of Amidol in 20 % $NaHSO_3$ solution were added with thorough shaking after each addition. After 10 min. the extinction was measured in a Klett-Summerson photometer with filter 62 (620 mμ). The values were unchanged up to 2 hours after the addition of Amidol. The phosphate concentrations were read from a standard curve. Orthophosphate was determined by adding perchloric acid and the other reagents to the unburnt sample and reading the extinction in the photometer.

For the nitrogen estimations the samples were burnt with 0.3 ml H_2SO_4 (diluted 1 : 1). The combustion was catalysed with 30 % H_2O_2 and carried out in pyrex tubes immersed in a salt bath at 180–200° C. After combustion the samples were diluted to 20 ml with distilled water. An aliquot of 1 ml of this solution was diluted with 5 ml distilled water and 1 ml Nessler reagent prepared by the method of KOCH and MCMEEKIN (11) was added. The extinction at 430 mμ was read in a Beckman photometer. The nitrogen content was read from a standard curve.

An egg suspension of the composition recorded above was divided into equal parts in a number of tubes. $CaCl_2$ solution was added to the suspensions to give the desired Ca concentrations and the suspensions were homogenized. After centrifugation, the control homogenate without Ca gave a colourless, almost clear supernatant. The residue showed quite a strong red colour, one layer being especially pigmented. In experiments where Ca ions were present in concentrations exceeding 0.000175 M during the homogenization, the supernatant was bright red and clear. The residue was also pigmented but not as strongly as in the control. In phosphate

---

[1] The TCA-insoluble fraction is the difference between the value for the supernatant and the TCA-soluble fraction.

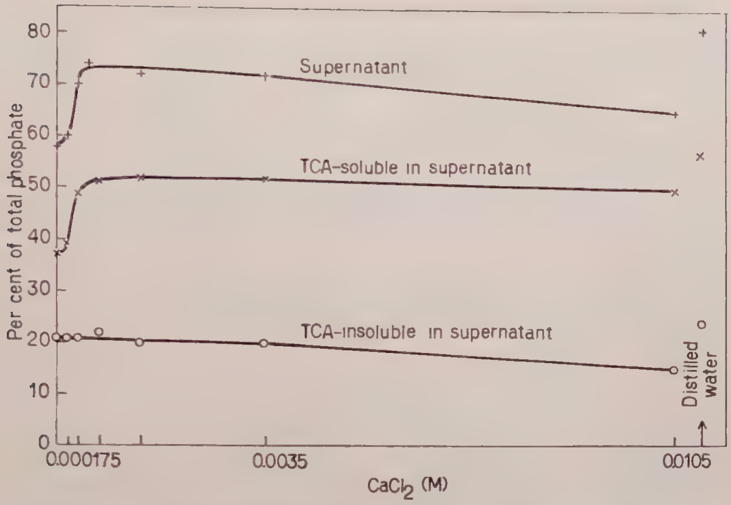


Fig. 1. The solubility of phosphate fractions in homogenates of unfertilized sea-urchin eggs prepared in isotonic NaCl with different concentrations of $CaCl_2$.

determinations on the different fractions, it was found that the supernatant contained more phosphate when calcium was present during the homogenization. The same observation was made for the nitrogen content. These changes in phosphate and nitrogen solubility appear to occur at the same calcium concentration as corresponds to the change in colour of the supernatant. Figs. 1 and 2 show that the changes in solubility of phosphorus and nitrogen in the presence of calcium have parallel courses. Nevertheless, the components of which the solubilities are thus altered are of quite different natures. The phosphate-containing fraction is TCA-soluble, while the increase in the solubility of nitrogen can be wholly attributed to a high-molecular component which is insoluble in TCA.

The above-described experiments were performed at room temperature. In order to establish whether the calcium effect could be inhibited by lowering the temperature, a series was carried out at 0° C. The same egg suspension was homogenized at room temperature (18° C) in order to eliminate biological variation and to permit a direct comparison of the changes in solubility of the phosphate fractions at the different temperatures. Table I shows that the reduction in temperature has no effect on the liberation of the low-molecular phosphate fraction brought about by treatment with calcium. There is, however, a reduction in the solubility of the high-molecular phosphate at the lower temperature when calcium is present in the homogenate. In these experiments the supernatants were also analysed for orthophosphate and it was found that the increase in the solubility of the TCA-soluble fraction could be wholly attributed to orthophosphate.

Experiments were also made in which the eggs were cytolysed with distilled water. It was found that the solubilities of the phosphorus and nitrogen fractions increased in the same manner as in the experiments with calcium. It will be seen in Figs. 1 and 2, however, that the increases in solubility were somewhat greater. The effect of calcium ions appears to be rather specific, since in experiments where

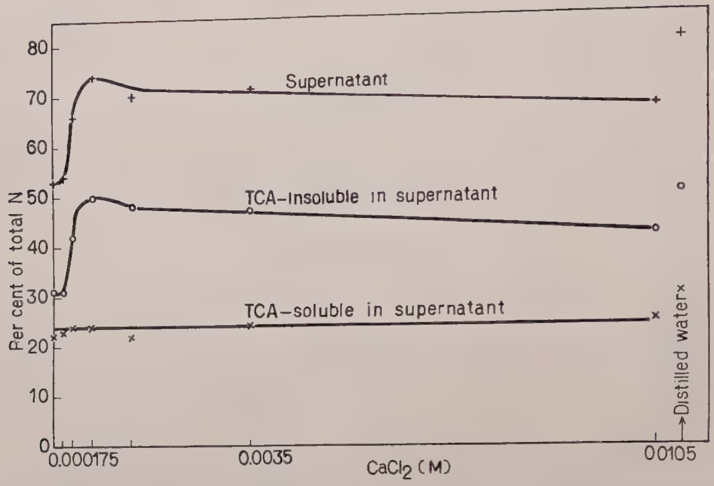


Fig. 2. The solubility of nitrogen fractions in homogenates of unfertilized sea-urchin eggs prepared in isotonic NaCl with different concentrations of $CaCl_2$.

magnesium replaced calcium no increases in solubility could be demonstrated. Nor were changes in solubility found when calcium was added after the homogenization.

The possible effect of hypertonic conditions on the distribution of the phosphate fractions was studied by subjecting the eggs to hypertonic media for different periods in the manner described by JUST (10). After the treatment the eggs were washed repeatedly with 3.8 % NaCl solution and then homogenized. After centrifugation, analysis of the different fractions showed that phosphate was liberated in the same manner in homogenization after hypertonic treatment as after homogenization in the presence of calcium. Table II shows that this effect was evident after hypertonic treatment for only 5 min.

Intact eggs were treated and homogenized in the manner described above. After centrifugation, the first supernatant was frozen with a mixture of dry ice and acetone. The residue was washed once with 3.8 % NaCl solution and centrifuged. The second supernatant and residue were then frozen. The different fractions were then frozen-

Table I. Effect of temperature on the solubility of different phosphate fractions. Solubilities are expressed as percentages of total P in the homogenate.

Temperature at homogenization	Conc. $CaCl_2$ molar	Supernatant			
		Total P	TCA-insoluble P	TCA-soluble P	TCA-soluble orthophosphate
18°C	0	64	22	42	22
	0.00175	77	19	58	38
	0.0035	77	21	56	37
0°C	0	65	25	40	22
	0.00175	67	11	56	37
	0.0035	69	13	56	37

Table II. Changes in phosphate solubility in a homogenate of eggs treated with hypertonic sea-water. Solubilities are expressed as percentages of total P in the homogenate.

Time of hypertonic treatment	Supernatant	
	Total P	TCA-soluble P
0 min.	54	33
5 "	65	47
15 "	67	46
30 "	68	50

Table III. The distribution of phosphate fractions expressed as percentages of total phosphate in the homogenate.

Fraction	Total	Orthophosphate	"ATP-phosphate"
First supernatant	52.0	14.5	9.7
Second supernatant	7.4	5.4	1.6
Residue	40.6	19.7	2.2

dried and pulverized. The distribution of the total phosphate among these three fractions was determined, whereafter a part of the frozen-dried material was extracted with cold 10 % TCA solution. This extract was analysed with respect to orthophosphate and phosphate, which was released after hydrolysis with boiling NHCl for 10 min. The results are given in Table III where it can be seen that the orthophosphate is equally divided between the residue and the combined supernatants. The easily hydrolysable phosphate corresponding mainly to the "ATP-phosphate", however, is differently distributed: by far the greater part is found in the supernatant and only a small amount remains in the residue.

2. Experiments with ribonuclease

In determinations of enzymic activity, use was made of homogenates of fresh eggs and extracts of frozen-dried material. These were prepared by the methods previously described. The enzymic activity was determined by the methods of Kunitz (12). Ribonucleic acid that is not depolymerized by incubation with the enzyme is precipitated with a solution of 0.25 % uranyl acetate in 2.5 % TCA. The amount of nucleic acid rendered acid-soluble during the incubation is taken as a measure of the enzymic activity.

The enzyme was extracted from the frozen-dried material by dialysing a suspension in 0.0005 NHCl against the same concentration of acid in the cold room for two days, the outer fluid being changed three times.[1] With extracts purified in this

---

[1] In our first experiments the frozen-dried material was extracted with distilled water, and when the aqueous extract was dialysed against 0.0005 NHCl; a very heavy precipitate was formed, containing among other things, the red pigment which had been dissolved in the water. This precipitate was devoid of enzymic activity. Atahigher acid concentration the precipitate was dissolved again.

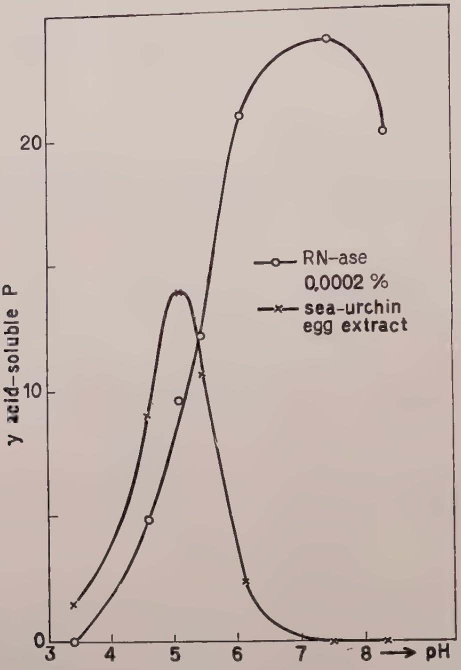


Fig. 3. Activity of crystalline ribonuclease and sea-urchin egg extract at different pH values.

manner, the pH optimum of the enzyme was determined, veronal-acetate mixtures being employed as buffers. The substrate was ribonucleic acid dissolved in distilled water and adjusted to pH 5.0 with dilute alkali. The time of incubation was 2 hours at 37° C. It will be seen from Fig. 3 that the sea-urchin enzyme shows a pH optimum at 5.2, while that of a commercial crystalline enzyme lies at pH 7–8. This latter value is also reported by Kunitz for pancreatic ribonuclease.

When using the method described above, Kunitz (12) reported that as much as 60 % of the ribonucleic acid could not be depolymerized. The part of the nucleic acid molecule unaffected by ribonuclease has been termed the core. In order to establish whether the enzyme of the sea-urchin egg left a part of the substrate molecule undegraded, we recorded a time curve for the enzymic activity. This curve, reproduced in Fig. 4, shows that only about 10 % of the ribonucleic acid was not broken down to nucleotides. The figure also shows that no orthophosphate was liberated in the depolymerization of the acid. It was demonstrated in further experiments that the degradation of nucleic acid was proportional to the amount of extract added.

It was of interest to determine whether the ribonuclease activity was present in the supernatant or bound to the residue. Experiments were therefore carried out with the same material as was employed for the study of the distribution of the phosphate fractions in the egg (Table III). The fractions "first supernatant" and "residue" were extracted during dialysis in the manner described above. The activities were then measured. The results show that all the activity was present in the residue. In experiments with fresh material, it has been found that the fraction

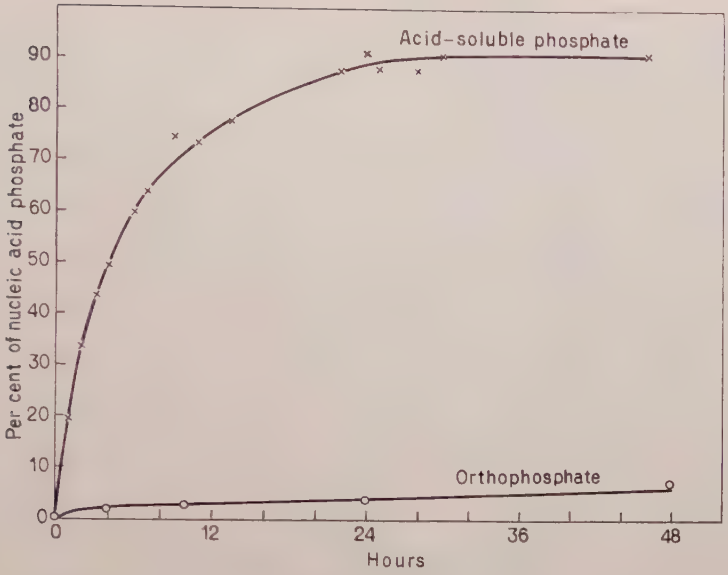


Fig. 4. Depolymerization of ribonucleic acid at pH 5.2 and 37°C.

bearing the enzymic activity can be altered if the homogenization is carried out in the presence of Ca, when the enzyme passes into the supernatant. This phenomenon is quite analogous to the liberation of phosphate and nitrogen fractions under the influence of Ca.

The possible effect of osmotic pressure on homogenates was investigated by treating homogenates in 3.8 % NaCl solution of fresh eggs with salt solutions of different concentrations. 1 ml portions of homogenate were mixed with 5 ml of sodium chloride solution varying in concentration from 0 to 8.5 %, so that a series of diluted homogenates of increasing NaCl concentration was obtained. After the homogenates had stood for 2 hours in the refrigerator, their enzymic activities were determined in the manner described above. The results are given in Fig. 5, where it can be seen that the activity is high at the lower salt concentrations, but gradually falls off with increasing concentration to reach a minimum at 3.8 % NaCl. This is a clear indication that the ribonuclease is enclosed in granules which are destroyed under hypotonic conditions.

According to Lamanna and Mallette (14), Mg ions have an inhibitory effect on ribonuclease. We have been unable, however, to demonstrate such an effect upon the ribonuclease from the sea-urchin egg. A further type of inhibitor for ribonuclease is reported by Roth (21, 22) to resemble heparin or a heparin-lipoprotein complex. Enzymic activities were therefore determined in the presence of 0.05 and 0.025 % heparin. Table IV shows that the enzyme was inhibited to about 28 % in the presence of the higher heparin concentration.

Roth (21) has reported that a factor inhibiting liver ribonuclease is present in liver homogenates. We investigated the possibility that such a factor might occur in the eggs by using suspensions of frozen-dried sea-urchin material. Roth showed that the inhibitors could be inactivated with lead. The addition of lead ions to the

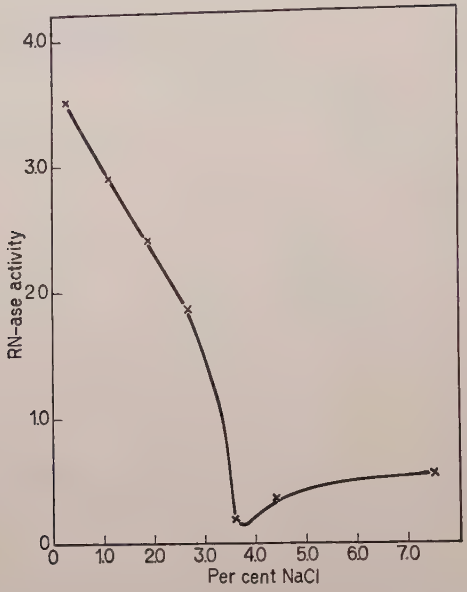


Fig. 5. Ribonuclease activity of homogenate in 3.8 % NaCl solution diluted with NaCl solutions of different concentrations.

Table IV. Inhibition of ribonuclease activity at different heparin concentrations.

Heparin conc. per cent	RN-ase activity	Inhibition per cent
0	14.2	0
0.025	12.0	16
0.05	10.2	28

suspensions during the determination of the enzyme activity did not appear, however, to affect the results. Copper, on the other hand, is a powerful inhibitor of the sea-urchin enzyme, and ROTH has shown that the liver ribonuclease is also strongly inhibited by this ion.

Discussion

The method employed in the present investigation, namely the fractionation of the egg into a supernatant and a residue, may give some information concerning the partition of different components between the different elements of the intact cell. The ground cytoplasm is represented by the supernatant, while the granules are present in the residue. Such a division of the cell may, however, give misleading results, as HOLTER (7) has shown in a recent study of a proteolytic enzyme in the amoeba *Chaos chaos*. On centrifuging these amoebae by a method introduced by HARVEY (4), he has demonstrated that an enzyme capable of splitting casein is present in the "heavy half", or that part of the cell in which granules are accumulated under the action of the centrifugal field. This suggests that the proteolytic enzyme is associated with the granules. If, however, the technique of centrifugal separation after homogenization is used the enzyme is found in the ground cytoplasmic fraction, which indicates that the enzyme is situated in the ground cytoplasm and is not present in the granules. From these findings, HOLTER draws the interesting conclusion that the proteolytic enzyme of *Chaos chaos* is associated with the granules *in vivo*, but that when the cell is subjected to violent treatment, such as homogenization, the enzyme is released from its natural site, so that it appears in the cytoplasmic fraction in the subsequent centrifugation.

In the sea-urchin egg, according to LUNDBLAD (18), there are proteolytic enzymes which can be activated by Ca and cysteine. This author has also shown that a proteolytic enzyme, with a pH optimum at 6–7, is activated in connection with the fertilization. HULTIN (9) found that the viscosity of a homogenate of a very dense egg suspension increased on addition of Ca. If, however, the homogenate was diluted, the increase in viscosity on subsequent addition of Ca was not so marked: on dilution beyond a certain point, indeed, the effect was completely absent. He suggested that these phenomena could be explained by the presence of a proteolytic enzyme system in the ground cytoplasm.

We have obtained analogous results in our experiments on the phosphate fractions of the egg. If the eggs were homogenized in a large quantity of isotonic NaCl containing Ca, acid-soluble phosphate was liberated into the supernatant fraction. This did not occur, however, when the homogenization was carried out under similar conditions but in the absence of Ca. Nor did addition of calcium to the latter homogenate result in the liberation of phosphate. The phosphate fraction released when the eggs are homogenized in the presence of Ca, is located in a particulate fraction of the egg. It may be conceived that these particles are broken down by a Ca-activated enzyme system. The extraordinary effect of Ca, despite the rapid dilution during the homogenization, might suggest that the enzyme system is not, situated in the ground cytoplasm, but is associated with the particulate fraction, which is broken down when the enzyme is activated with Ca. When the eggs are homogenized in Ca-free isotonic salt solution, the enzyme may be released from the particles in the same manner as the protease of *Chaos chaos* (cf. HOLTER), so that a later addition of Ca will be without effect: the enzyme has been released from its substrate and can no longer degrade the particles. The fact that the main part of the phosphate, hydrolysable by boiling with N HCl for 10 min., was found in the supernatant does not necessarily mean that this phosphate fraction entirely originated in the ground cytoplasm in the intact egg. As with the proteinase, there remains a possibility that the phosphate fraction or a part of it in vivo is associated with the particulate fraction, but when the cells are homogenized the phosphate fraction is released and appears in the supernatant.

The particulate fraction degraded on homogenization of the eggs in the presence of Ca, contains a ribonuclease in addition to acid-soluble phosphate. In the surrounding ground cytoplasm there is, among other things, a high-molecular phosphate fraction which presumably derives from ribonucleic acid or ribonucleoprotein. When the particles are broken down by the Ca-activated enzyme, the enclosed nuclease will be liberated and gain access to its substrate, so that acid groups are liberated. Such a liberation of acid groups on addition of Ca to homogenates has, indeed, been demonstrated by HULTIN in Warburg experiments. This may be of interest in relation to the acid formation shown by RUNNSTRÖM (25) to be associated with fertilization and also with regard to the fact that fertilized eggs are more basophile than unfertilized. The release of orthophosphate from the granules may also contribute to the acid formation.

In experiments with mammalian ribonuclease, ROTH (22) has shown that heparin has an inhibitory action on this enzyme. We have shown that the same is the case with the ribonuclease of the sea-urchin egg. HARDING and HARDING (3) found that heparin inhibited fertilization, but that the cleavage of the egg was not inhibited if heparin was added to an egg suspension only 4–6 seconds after fertilization. According to an investigation by PAFF *et al.* (20), ribonucleic acid accumulates in cells grown in the presence of heparin. Cell division in these experiments occurs very slowly, if at all, and this is taken by the authors to indicate that an inhibition of the intracellular ribonuclease prevents the catabolism of ribonucleic acid and hence inhibits cell division. HARDING's finding that cell division is not inhibited if heparin is added 4–6 seconds or later after the moment of fertilization, does not exclude the possibility that, as PAFF *et al.* suggest, the ribonuclease in the egg partakes in the catabolism of ribonucleic acid: it may well be that the permeability of the egg surface is so altered after fertilization that heparin can no longer penetrate to the interior.

In the experiments carried out at 0° C in the present investigation, certain differences in solubility were observed in homogenates prepared in the presence and absence of calcium. RUNNSTRÖM and KRISZAT (25), using the centrifuge microscope, have shown that Ca exerts some effect upon intact eggs, in that the rigidity of the cytoplasm is reduced if the Ca concentration in the surrounding medium is increased to double the normal value. It may be of some interest in this connection to mention the results obtained by LINDVALL and CARSJÖ (16) in experiments in the precipitation with Ca of a nucleoprotein from extracts of frozen-dried sea-urchin eggs. A maximal precipitation was observed at a concentration of 0.0125 M$CaCl_2$, lower yields being obtained at higher or lower concentrations.

The differences in solubility observed in homogenates must be of a subtle nature, since they cannot be detected if the homogenization has been performed at room temperature. The differences found in frozen-dried material, on the other hand, are evident even if the extraction is allowed to proceed at room temperature (17). In experiments with nucleoprotein isolated from bacteria treated with ultrasonics, SEVAG and SMOLENS (26) have shown that this material cannot be precipitated with Ca ions before it has been treated with acid or alkali. The differences between fresh and frozen-dried sea-urchin eggs may lie in the degree of alteration of the cytoplasmic nucleoprotein, upon which may depend the affinity of this substance for Ca.

SUMMARY

On the homogenization of a suspension of sea-urchin eggs Paracentrotus lividus in 3.8 % NaCl solution, where calcium ions are present in concentrations exceeding 0.000175 M, there occurs a breakdown of a cytoplasmic particulate fraction from which the release of an orthophosphate, a high-molecular nitrogen fraction and a ribonuclease was observed.

Calcium can produce this breakdown at 0° C as well as at 18° C. At the lower temperature there is a decrease of the solubility of a high-molecular phosphate fraction in presence of calcium.

If calcium is added after homogenization, no such particulate breakdown occurs. Calcium cannot be replaced by magnesium.

In unfertilized eggs homogenized in NaCl solution the orthophosphate is present in equal amounts in particles and ground cytoplasm. The main part of the phosphate hydrolysable by boiling with N HCl for 10 min., is found in the ground cytoplasm.

The ribonuclease of the unfertilized egg has its pH optimum at 5.2. This enzyme is bound to the particulate fraction and can be released by treating these particles with NaCl solutions of lower concentration than 3.8 %.

The activity of the enzyme is not affected by magnesium while copper and 0.05 % heparin have an inhibiting effect.

Acknowledgements

We wish to express our deep gratitude to Professor J. Runnström, Director of the Wenner-Gren Institute, for never failing support and interest in our work. We are very indebted to Professor R. Dohrn and his staff at the Zoological Station in Naples for laboratory facilities and material.

The investigations were supported by grants from the Swedish Natural Science Research Councils.

The Wenner-Gren Institute for Experimental Biology, Stockholm.

REFERENCES

1. Allen, R. J. L., Biochem. J. 34, 858 (1940).
2. Bernstein, M. H., Biol. Bull. 97, 255 (1949).
3. Harding, C. V. and D. Harding, Arkiv Zool. 3, 357 (1952).
4. Harwey, E. N., J. of the Franklin Institute 214, 1 (1932).
5. Heilbrunn, L. V., Colloid Chemistry of Protoplasm. Protoplasma Monographien 1, Berlin, 1928.
6. —— An outline of General Physiology, W. B. Saunders Co., Philadelphia and London, 1937.
7. Holter, H., Nature 171, 815 (1953).
8. Hultin, T., Exp. Cell Research 1, 160 (1950).
9. —— Exp. Cell Research 1, 272 (1950).
10. Just, E. E., Biol. Bull. 43, 383 (1922).
11. Koch, F. C. and T. L. McMeekin, J. Am. Chem. Soc. 46, 2066 (1924).
12. Kunitz, M. J., J. Gen. Phys. 24, 15 (1940).
13. Laki, K. and A. Clark, Arch. Bioch. 30, 187 (1951).
14. Lamanna, C. and M. F. Mallette, Arch. Bioch. 24, 451 (1949).
15. Lindberg, O., Exp. Cell Research 1, 105 (1950).
16. Lindvall, S. and A. Carsjö, Exp. Cell Research 2, 491 (1951).
17. —— Unpublished data.
18. Lundblad, G., Arkiv Kemi 4, 537 (1952).
19. Mirsky, A. E., Science 84, 333 (1936).
20. Paff, G. H., Sugiura, H. T., Bocher, C. A., and J. S. Roth, Anat. Record 114, 499 (1952).
21. Roth, J. S., Nature 171, 127 (1953).
22. —— Arch. Bioch. 44, 265 (1953).
23. Runnström, J., Protoplasma 5, 201 (1928).
24. Runnström, J. and G. Kriszat, Exp. Cell Research 1, 284 (1950).
25. Runnström, J., Arkiv Zool. 21 B, No. 8 (1930).
26. Sevag, M. G. and J. Smolens, J. Biol. Chem. 140, 833 (1941).
27. Spicer, S. G., J. Biol. Chem. 190, 251 (1951).

Tryckt den 4 mars 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 3

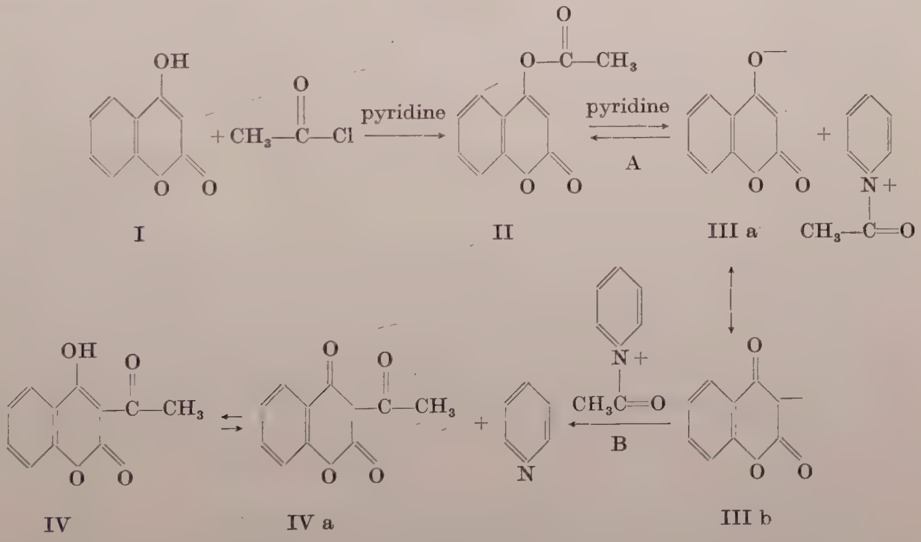
Communicated 28 October 1953 by ARNE FREDGA

Some remarks on the mechanism attributed to the reaction of 4-hydroxycoumarin with acetyl chloride

By ARNE BRÄNDSTRÖM

In a recent paper on the acetylation of 4-hydroxycoumarin (I), EISENHAUER and LINK (1) give a mechanism for the rearrangement of 4-acetoxycoumarin (II) into 3-acetyl-4-hydroxycoumarin (IV). Since this is a good example of the rearrangement of an enolester into a C-acyl compound, a very important reaction from both theoretical and practical points of view, a few supplementary remarks may be of interest.

The mechanism given by the above authors is well illustrated by the following formula:



In this reaction it was proved that the reaction *A* was rapid and reversible, whereas the reaction *B* was slow and irreversible. From this the authors draw the very remarkable conclusion that the slow step in the reaction *B* was probably the formation of the carbanion (III b), for which they suggested the term "excited state" in preference to "resonance hybrid" or "canonical form". Such a nomenclature would be very unfortunate, however, as the term "excited state" has been used for a long time to designate high energy states differing from the ground state in the kind of hybridisation concerned, and sometimes in the steric positions of the atoms (2, 3). These states have been found very useful in predicting the light absorption and colour of organic substances. The energy necessary to excite a compound such as the anion III to a state of this kind must be much too high for a reaction involving this state to be of any kinetic importance. The absorption of visible light corresponds to energies in the region 30–90 Cals./Mol., ultraviolet to still higher energies.

The assumption of "excited states" is by no means necessary in explaining why the formation of 3-acetyl-4-hydroxycoumarin (IV) is a slow process whereas the formation of 4-acetoxycoumarin (II) is a very rapid one.

If the electronic state of the anion III is considered, it must be remembered that it is a typical mesomeric anion with π-orbitals extended over all atoms in the anion. The electron density at different points in this anion is given by the $|\Psi|^2$ function, which is a fundamental property of the anion and can only be changed by very high energies, or by the attraction of other atoms to it (6). From the experimental and theoretical data available for such anions it can be concluded that the mesomeric anion III has a very high electron density at the oxygen atom in the 4-position and a rather high electron density at the carbon atom in the 3-position. In the reaction of the anion III with cationoid reagents the most assailable positions will be these two places of high electron density. In the illustration of each of these two possibilities, the anion III is represented by the two formulae III a or III b, but it is very important to remember that they represent the same anion, and that conclusions about a slow transformation of one of them into the other indicate a misunderstanding of the principle of the quantum theory of organic reactions, especially as such a transformation would involve electrons only and must be extremely rapid.

The point at which a cationoid reagent attacks a mesomeric anion will depend on the electron density of the anion, the energy of activation, and the steric effects for the different processes.

In the anion III the electron density factor will greatly favour an attack at the oxygen atom.

Regarding the energy of activation, DEWAR (4) has concluded that it is higher for the O-substitution than for the C. As he achieved this result by comparing the total binding energies of a ketone and the corresponding enol, without remembering the very important fact that bond energies are not additive for conjugate systems (5), this conclusion about the activation energies cannot be accepted without further enquiry. The present author (7) has shown from available experimental results that the difference in the energy of activation for the two different processes is very small.

The steric effects are very hard to predict, but it seems to be no reasons to expect a C-acylation reaction of the anion III to be sterically more favoured than an O-acylation one.

The result is that no factor favouring the C-acylation reaction of the anion III can compensate for the very unfavourable electron distribution, as compared with the O-acylation reaction. We can thus expect a rapid O-acylation to occur, together with a slow C-acylation. Since the O-acylation is reversible, there will be a slow accumulation of the C-acylation product if the reaction time is extended.

EISENHAUER and LINK (1) found that 4-methoxycoumarin did not give any 3-acetyl-4-methoxycoumarin when treated with acetyl chloride in pyridine, and mentioned this fact as an argument that a direct C-acylation does not take place in the case of 4-hydroxycoumarin. Such a conclusion seems very curious if we recall that 4-methoxycoumarin is not present as an anion in a pyridine solution, while 4-hydroxycoumarin is, and the comparison of the two compounds in this reaction therefore tells us nothing about the mechanism. As an analogous case it may be mentioned that ethyl methoxycrotonate does not react with acetyl chloride, whereas ethyl hydroxycrotonate (ethyl acetoacetate) in the form of the sodium compound readily gives the C-acetyl compound by a direct C-acylation (9).

Finally, it may be concluded from the experimental results given by EISENHAUER and LINK (1) that they have no evidence for the suggestion that the acetylpyridinium cation is the acetylation agent in the C-acetylation reaction. It must be remembered that enol acetates are good acetylation agents, and may quite well function as such in this reaction. Many examples of such reactions are known, but only one will be given here. If ethyl acetoxycrotonate is treated with the sodium compound of ethyl benzoylacetate, some ethyl acetylbenzoylacetate is formed (10).

In the reactions in a pyridine solution, it is probable that the formation of enol acetates and acetylpyridinium cations are both sufficiently rapid reactions, so that neither acylation reagent can definitely be stated to be the actual acylation reagent.

In this connection it is interesting to note that even acyl-groups directly attached to carbon atoms may have a very high mobility (8).

Laboratory of Organic Chemistry, University of Uppsala, Sweden.

REFERENCES

1. H. R. EISENHAUER and K. P. LINK, J. Am. Chem. Soc. *75*, 2044 (1953).
2. C. K. INGOLD and G. W. KING, J. Chem. Soc. *1953*, 2702.
3. M. J. S. DEWAR, The electronic theory of organic chemistry, Oxford 1949, p. 296.
4. —— ibidem p. 104.
5. —— ibidem p. 33.
6. A. BRÄNDSTRÖM, Arkiv f. Kemi *6*, 168 (1953).
7. —— ibidem p. 165.
8. —— ibidem p. 201.
9. A. MICHAEL, Ber. *38*, 2088 (1905).
10. W. DICKMANN and R. STIEN, Ber. *37*, 3395 (1904).

Tryckt den 4 mars 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 4

Communicated 9 Sept. 1953 by J. ARVID HEDVALL

Kinetic study of the zirconium silicate/calcium oxide reaction

By DEMETRIO A.-ESTRADA

With 4 figures in the text

Summary

With the present paper the author hopes to contribute to the kinetic study of the transposition reactions between ortho-silicates and alkaline-earth oxides, taking the case of zirconium ortho-silicate and calcium oxide into special consideration.

Tests were carried out with pulverized mixtures of the reacting substances of controlled grain size and the development of the reaction studied chemically and by X-ray methods.

Variation of yield as a function of time at temperatures between 1100 and 1400° C has been studied and a rapid increase of yield during the first phases of calcination was observed while later rises took place slowly.

With respect to the variation of yield with temperature it was shown that by increasing the temperature from 1200 to 1300° C, the yield was increased more rapidly. At the same time a rapid formation of free zirconium oxide occurred.

Introduction

The great technical interest offered by zirconium oxide, pure or mixed with other oxides, on account of its refractory properties and its special behaviour at high temperatures as regards emission of light, and its present wide use in special compositions for porcelain and opacifiers for ceramic enamels, have induced us to pay special attention to that point of the chemistry of solid state. As on the other hand Spain exploits monazitic sand deposits having considerable zirconium silicate content, and since we have succeeded in purifying this product by work carried out by us in a previous stage, it was decided to study a type of reaction that might give alkaline-earth silico zirconates and zirconium oxide. For this purpose the zirconium silicate/calcium oxide reaction has been chosen.

Method of investigation

The following products were used: calcium oxide, derived from the decomposition of calcium carbonate, Merck's reagent No. 2067. Zircon ore mineral from the Norwegian Brevig deposits which the Mineralogical Museum of Stockholm had placed at our disposal and which were purified by our method to 99,7 % $ZrSiO_4$.

The grain size of the basic materials was controlled by centrifuging in a Bahco powder centrifuge type SSP, and tested by microscopic observations. Zirconium silicate was separated in sizes between 16 and 21 μ, and calcium oxide in particles smaller than 5 μ.

Experiments were carried out on pellets of 8 mm Ø and 2 mm thickness, obtained from an equimolecular mixture of both products compressed at 12 tons/cm², using 8 % of glycerol as binder.

For all experiments these pellets, supported on platinum boats, had been calcinated in a tubular kanthal coil or platinum furnaces which had previously been stabilized at calcination temperature. Temperatures were measured with a 10 % Pt-PtRh thermo-couple joined to a millivoltmeter; the deviations observed in the temperature range employed were ± 5° C.

After the pellets were calcinated at the reaction temperature for a fixed time, they were taken out of the furnace and cooled in a desiccator containing silica gel. Then they were carefully ground in an agate mortar, and with this material the analysis of the reaction products was carried out.

The following direct chemical determinations were carried out: free calcium oxide, by W. Jander and E. Hoffmann's method (1), using glycerol and methanol. This method was also employed to determine calcium oxide combined as calcium zirconate and calcium silicate, which were decomposed in boiling concentrated hydrochloric acid. Zirconium oxide, combined as calcium zirconate and liberated in the hydrochloric solution, was determined by C. A. Kumins' procedure (2), using a solution of mandelic acid as precipitating agent.

At the same time, to confirm the results of the chemical analysis, the reaction products were studied by X-rays.

Experiments were carried out at a temperature range from 1118 to 1425° C, the reaction time for each test being suitably varied.

Progress of the reaction

Zirconium silicate particles have a considerably larger diameter than calcium oxide, and microscopic observation has shown a predominance of polyhedrical form with many faces. We, therefore, regard the system as formed by large spheres of one of the components totally surrounded by the finest particles of the other. Thus a reaction surface can be assumed that is equivalent to the area of the zirconium silicate "spheres".

When an experiment is carried out at a given temperature and the variation of yield as a function of the reaction time is observed, it can be noticed that at first the reaction progresses rapidly but becomes slower afterwards. In our case of solids in contact, this can be explained in the following manner: during the first moment direct contact exists between the reacting phase (Fig. 1 a), but once the process has begun, a reaction film of the composition AB (Fig. 1 b) is formed. The diffusion process has to continue through the film either in the direction of AB or of BA. In order to determine the direction of the diffusion radioisotopic techniques have to be used. Since we did not employ them, we cannot make any statement about the direction.

From the analytical results obtained in each test, we can state that calcium oxide replaces zirconium oxide in the silicate with relative facility, originating ortho- or meta-calcium silicate, or a mixture of both, according to the composition

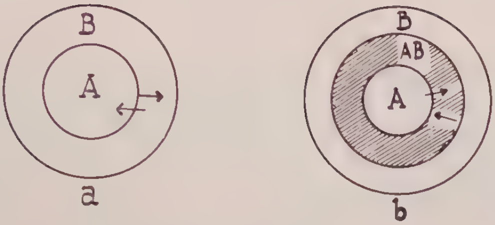


Fig. 1.

and the calcination temperature. Zirconium oxide, on account of its acid character, could dissolve any free calcium oxide present in the system, forming solid solutions. Where the calcium oxide is insufficient, the freed zirconium oxide tends at first to combine with the calcium meta-silicate formed to give a calcium silico-zirconate. The reaction, in a ratio of 1:1 is as follows:

$$ZrSiO_4 + CaO \rightarrow CaSiO_3 + ZrO_2. \tag{1}$$

This simple reaction does not take place directly in actual practice, but rather in several intermediate phases as the temperature rises.

As can be seen in the X-ray diagram, the X-ray analysis of pellets calcinated at 1118° C shows the presence of zirconium silicate and combined zirconium oxide which proves that at this temperature the formation of calcium silico-zirconate has already begun. (Fig. 2.)

At 1205° C the X-ray analysis shows for the first time the presence of free zirconium oxide, whilst the original amount of zirconium silicate considerably decreased, and that of silicozirconate increases. At the same time it can be deduced from the results obtained by chemical analysis that calcium ortho-silicate is present. All this leads us to suppose that the reaction is starting as follows:

$$2ZrSiO_4 + 3CaO \rightarrow ZrCaSiO_5 + Ca_2SiO_4 + ZrO_2. \tag{2}$$

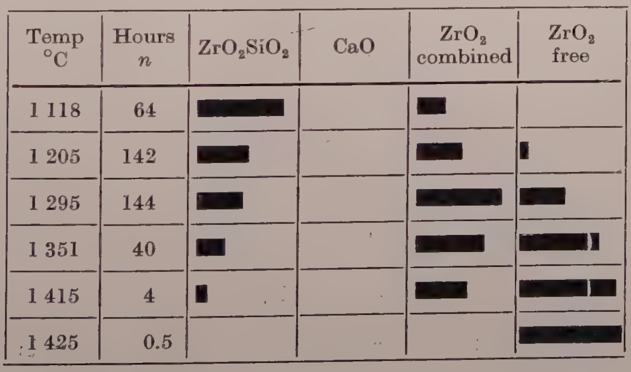


Fig. 2.

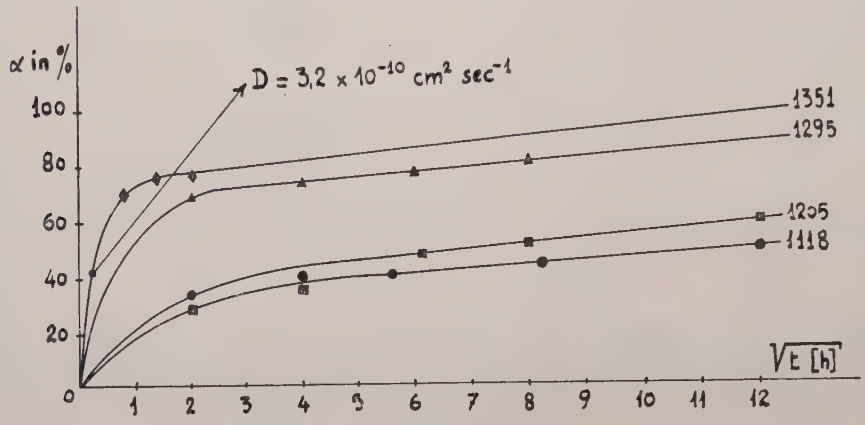


Fig. 3.

At 1295° C the X-ray diagram shows a considerable increase of silico-zirconate due to the progress of reaction (2) as well as to the reaction of formed calcium ortho-silicate with new zirconium silicate, according to:

$$ZrSiO_4 + Ca_2SiO_4 \rightarrow CaSiO_3 + ZrCaSiO_5. \quad (3)$$

Both the chemical and the X-ray analysis show from 1300° C a progressive reduction of the amount of silico-zirconate present, while at the same time the proportion of free zirconium oxide rises. These facts may be explained by the suggestion that from 1300° C the decomposition of silico-zirconate is quicker than its formation. This decomposition gives rise to new zirconium oxide, as follows:

$$ZrCaSiO_5 \rightarrow CaSiO_3 + ZrO_2. \quad (4)$$

At 1415° C there exist only traces of the original zirconium silicate, a small quantity of silico-zirconate and a large of free zirconium oxide. At 1425° C, the X-ray diagram only shows the presence of free zirconium oxide, all the other zirconium compounds having completely disappeared; these results were also obtained by chemical analysis.

X-ray examination of the products calcinated at temperatures higher than 1300° C does not show the presence of calcium silicates, whilst chemical analysis still shows their existence. This is due the fact that these silicates are in vitreous phase. The quick cooling the pellets have to undergo from the temperature of the experiment to room temperature can explain the phenomenon as "freezing in" of that state. On the other hand it was observed optically that samples calcinated at very high temperatures look like a crystalline mass joined by vitreous phase.

From Fig. 3, where the variation of the yield (referred to the decomposition of zirconium silicate) as a function of the square root of time is represented, we can assume that in the 1200–1300° C range a secondary reaction is initiated which conducts parallelly to the composition of zirconium silicate. This reaction consists, as already stated, in an attack of new zirconium silicate by calcium ortho-silicate,

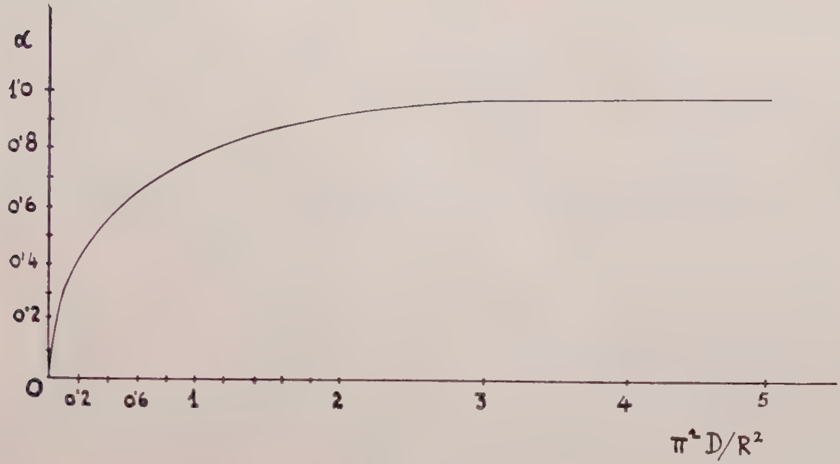


Fig. 4.

already present in the system at these temperatures, see scheme (3). At the same time a rapid formation of free zirconium oxide can be observed, according to chemical and X-ray analyses.

From the yield/time curve for the reaction at 1351° C calculations were made for the deduction of the diffusion constant. According to B. SERIN and R. T. ELLICKSON (3), this constant (if the crystals can be considered as spheres) is represented as a function of the yield by the equation:

$$\alpha = 1 - (6/\pi^2) \sum_n (1/n^2) \exp(-n^2 k t)$$

where $k = \pi^2 D/R^2$. The same authors have reproduced this equation (Fig. 4) and presented a curve where the yield α is related to k. From this curve the product kt can be deduced for a given yield and the average radius of the spheres ($R = 1 \times 10^{-3}$ cm) being known, by using the expression:

$$\frac{kt}{t} = \frac{\pi^2 D}{R^2}$$

the diffusion constant D for the process studied is directly derived. At 1351° C and for a reaction time $t = 56$ sec., the yield was $\alpha = 0.4$. The value of kt found graphically is $kt = 0.18$, the diffusion constant thus being

$$D = 3.2 \times 10^{-10} \text{ cm}^2 \text{ sec}^{-1}.$$

This value is only preliminary. The final results shall lead to the determination of the activation energies of the various partial reactions of the entire process.

I have to thank Prof. Dr. J. A. Hedvall for the interest shown in these experiments and Dr. R. Lindner for his help and guidance in the employment of the techniques used. Likewise I am obliged to the Consejo Superior de Investigaciones Científicas which has made possible my stay at the *Institutionen för Silikatkemisk Forskning* in Gothenburg and the *Statens Tekniska Forskningsråd*, which placed at our disposal the facilities for carrying out this study.

Institute for Silicate Research, Chalmers Technical University, Gothenburg.

REFERENCES

1. W. Jander und E. Hoffman, Angew. Chemie; *46* (1933) 78.
2. C. A. Cumins, Anal. Chem., *19* (1947) 376.
3. B. Serin and R. T. Ellickson, Determination of Diffusion Coefficients, Journ. of Chem. Phys. *9* (1941) 742.

Tryckt den 4 mars 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 5

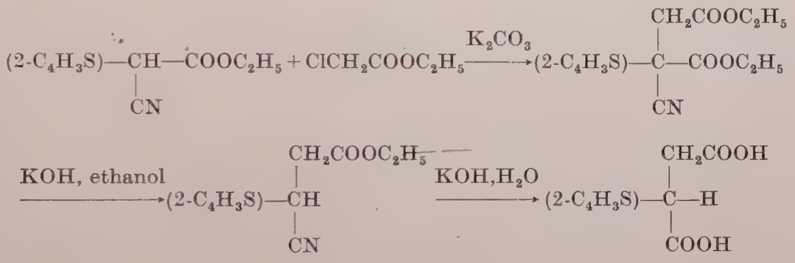
Communicated 28 October 1953 by ARNE FREDGA and KARL MYRBÄCK

Optical resolution and configuration of 2-thienylsuccinic acid

BY KURT PETTERSSON

Four general methods for the preparation of substituted succinic acids have been applied at this laboratory to the synthesis of 2-thienylsuccinic acid:

1. Ethyl 2-thienylcyanoacetate, ethyl chloroacetate and anhydrous potassium carbonate react in anhydrous acetone, giving ethyl 2-thienylcyanosuccinate (1). Decarboxylation (2) and alkaline hydrolysis of the product obtained yields 2-thienylsuccinic acid:

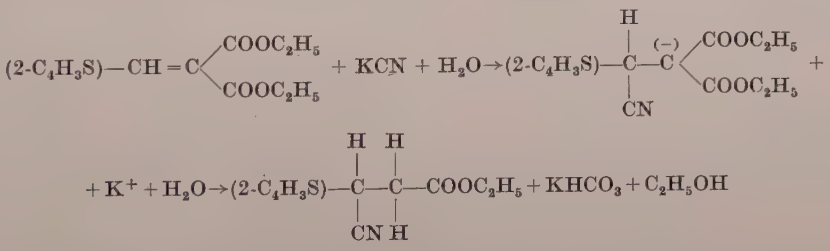


$$(2\text{-}C_4H_3S)\text{—}\underset{\displaystyle CN}{\underset{|}{CH}}\text{—}COOC_2H_5 + ClCH_2COOC_2H_5 \xrightarrow{K_2CO_3} (2\text{-}C_4H_3S)\text{—}\overset{\displaystyle CH_2COOC_2H_5}{\overset{|}{\underset{\displaystyle CN}{\underset{|}{C}}}}\text{—}COOC_2H_5$$

$$\xrightarrow{\text{KOH, ethanol}} (2\text{-}C_4H_3S)\text{—}\overset{\displaystyle CH_2COOC_2H_5}{\overset{|}{\underset{\displaystyle CN}{\underset{|}{CH}}}} \xrightarrow{KOH,H_2O} (2\text{-}C_4H_3S)\text{—}\overset{\displaystyle CH_2COOH}{\overset{|}{\underset{\displaystyle COOH}{\underset{|}{C}}}}\text{—}H$$

A modified method for the carbethoxylation of 2-thenyl cyanide (3) makes the method rather convenient.

2. Ethyl 2-thenalmalonate, obtained from 2-thiophenecarboxaldehyde (2-thiophenealdehyde) and ethyl malonate in the usual way, reacts with potassium cyanide in water, forming ethyl β-(2-thienyl)-β-cyanopropionate. Alkaline hydrolysis of the ester group gives β-(2-thienyl)-β-cyanopropionic acid, which on boiling in water undergoes decarboxylation, yielding 2-thienylsuccinic acid:



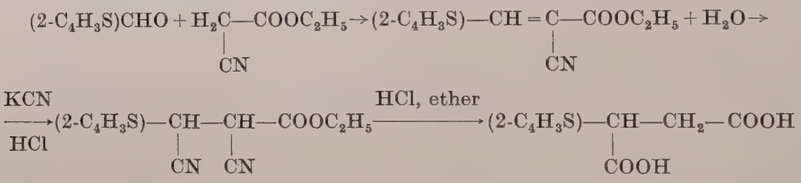
$$(2\text{-}C_4H_3S)\text{—}CH{=}C\left\langle\begin{array}{l}COOC_2H_5\\ COOC_2H_5\end{array}\right. + KCN + H_2O \rightarrow (2\text{-}C_4H_3S)\text{—}\overset{\displaystyle H}{\overset{|}{\underset{\displaystyle CN}{\underset{|}{C}}}}\text{—}\overset{(-)}{C}\left\langle\begin{array}{l}COOC_2H_5\\ COOC_2H_5\end{array}\right. +$$

$$+ K^+ + H_2O \rightarrow (2\text{-}C_4H_3S)\text{—}\overset{\displaystyle H}{\overset{|}{\underset{\displaystyle CN}{\underset{|}{C}}}}\text{—}\overset{\displaystyle H}{\overset{|}{\underset{\displaystyle H}{\underset{|}{C}}}}\text{—}COOC_2H_5 + KHCO_3 + C_2H_5OH$$

It is a common opinion that thiophene compounds are sensitive to strong acids, at least at higher temperatures. Acid hydrolysis should therefore be avoided, if possible. Strong alkaline solutions must also be avoided, for the analogous β-phenyl-β-cyanopropionate gives considerable amounts of cinnamic acid when it is treated with two moles of sodium hydroxide per mole of ester in order to obtain phenylsuccinic acid in one step (4). Now, WIDEQVIST (5) has stated, that β-cyanocarboxylic acids are readily hydrolysed in water solutions. Thus β-phenyl-β-cyanopropionic acid is converted at room temperature to phenylsuccinic acid (6). It seems reasonable to suppose, that the same would happen to the thiophene analogue obtained from ethyl β-(2-thienyl)-β-cyanopropionate by the aid of one mole of sodium hydroxide per mole of ester.

It is probable, however, that ethyl β-(2-thienyl)-β-cyanopropionate, analogously to ethyl β-(2-thienyl)-α,β-dicyanopropionate (*vide infra*), can be hydrolysed to 2-thienylsuccinic acid on standing for several days in a mixture of concentrated hydrochloric acid and ether.

3. 2-Thiophenecarboxaldehyde reacts with ethyl cyanoacetate in the presence of small amounts of piperidine, forming ethyl β-(2-thienyl)-α-cyanoacrylate. This substance adds potassium cyanide and yields, after acidification, ethyl β-(2-thienyl)-α,β-dicyanopropionate, which on standing for several days in a mixture of concentrated hydrochloric acid and ether is converted to 2-thienylsuccinic acid:

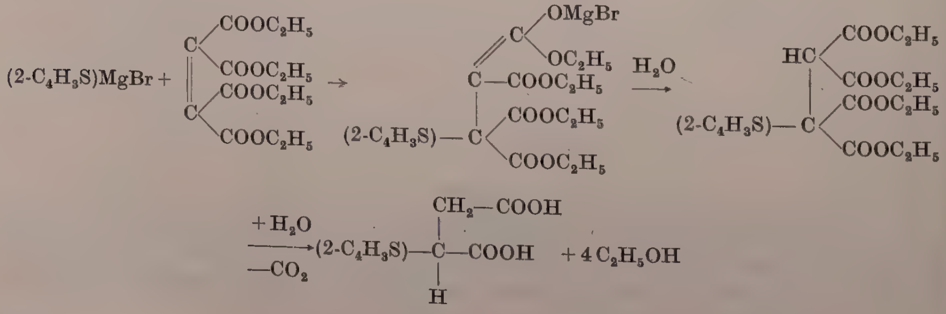


$$(2\text{-}C_4H_3S)CHO + \underset{\displaystyle CN}{H_2C}\text{—}COOC_2H_5 \rightarrow (2\text{-}C_4H_3S)\text{—}CH{=}\underset{\displaystyle CN}{C}\text{—}COOC_2H_5 + H_2O \rightarrow$$

$$\xrightarrow[HCl]{KCN} (2\text{-}C_4H_3S)\text{—}\underset{\displaystyle CN}{CH}\text{—}\underset{\displaystyle CN}{CH}\text{—}COOC_2H_5 \xrightarrow{HCl,\ ether} (2\text{-}C_4H_3S)\text{—}\underset{\displaystyle COOH}{CH}\text{—}CH_2\text{—}COOH$$

If ethyl cyanoacetate is not available, it is probably better to start with 2-thiophenecarboxaldehyde and potassium cyanoacetate. A preliminary proof shows, that β-(2-thienyl)-α-cyanoacrylic acid is then formed in a yield of about 50 %, and can be isolated as the sparingly soluble potassium salt. The acid may be esterified in the usual way and the ester treated as mentioned above. (Compare Org. Syntheses *I* (1946) pp. 181 and 451, dealing with the preparation of phenylsuccinic acid).

4. For the sake of completeness, a fourth method for the preparation of the acid may be mentioned. It has been used at this laboratory by Mr S. GRONOWITZ (7), who has kindly put his result at the author's disposal. He has prepared the acid from 2-thiophenemagnesium bromide and ethyl ethylenetetracarboxylate according to the method described by CHI-YI HSING and LIEN-TANG LI (9). These authors prepared phenylsuccinic acid, using a large excess of phenylmagnesium bromide. GRONOWITZ, however, used equivalent amounts of the reactants. The yield was about 25 %.



The usefulness of a particular method depends upon the starting materials. If 2-thenyl cyanide is available, the first method is very convenient. The preparation of this substance involves, however, the lachrymatory and unpleasant 2-thenyl chloride, which must be handled with care. The starting material in methods 2 and 3 is the readily available 2-thiophenecarboxaldehyde (8), prepared from thiophene and N-methylformanilide. Methods 2 and 3 are about equally convenient, 2 being more convenient if ethyl cyanoacetate is not available. Method 4 is merely of theoretical interest.

Optically inactive 2-thienylsuccinic acid crystallises from water as a granular, colourless powder, or if the solution is more dilute, in slightly yellow, hemispherical, hard crystal aggregates. After several recrystallisations from water it melts at 162°–163°. At the melting point a gas is evolved, which indicates that the acid is then converted to the corresponding anhydride.

In a preceding paper it was assumed that optically inactive 2-thienylsuccinic acid is dimorphic (1), like phenylsuccinic acid (10–11). Owing to the instability of the acid at the melting point, the question cannot be settled by a phase study in a hot stage microscope. An X-ray investigation is in progress and will be published later (13).

The primary alkaloid salts of the acid are apparently too soluble in the usual solvents to be employed in resolving the acid into the optical antipodes. Several crystallised secondary salts have been obtained, however, some of which possess strong resolving power. Cinchonidine in dilute ethanol and (−)-α-phenylethylamine in ethyl acetate give satisfactory yields of the (+)- and (−)-2-thienylsuccinic acids respectively.

(+)-*2-Thienylsuccinic acid* is obtained in a state of purity by recrystallisation from water of the crude acid liberated from the cinchonidine salt. It crystallises in colourless, transparent needles or rods, which may attain a length of several centimeters if the solvent is slowly evaporated. If a hot solution of the acid is allowed to cool rapidly, the acid is obtained as a granular, colourless powder. A similar behaviour of optically active phenylsuccinic acid is reported by WREN and WILLIAMS (12), who also found an instable modification, changing to the stable modification on standing in water for a night in a refrigerator. Investigations in a hot stage microscope lead FREDGA and MATELL to assume optically active phenylsuccinic acid to be dimorphic (11). Since it was possible, that (+)-2-thienylsuccinic acid might likewise exist in two modifications, the two above-mentioned preparations were examined with X-rays. The X-ray powder photographs were identical, however. Now, if this modification is heated above 125°, it changes to another modification, without melting or racemisation (13), indicating the acid to be at least dimorphic. Later it will also be shown that the "low temperature" modifications of the optically active 2-thienylsuccinic and phenylsuccinic acids are isomorphic (13).

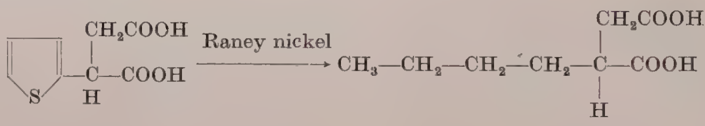
(+)-Thienylsuccinic acid melts at 158°–162°, with decomposition. As in the case of the optically inactive acid a gas is evolved, indicating anhydride formation, but in addition racemisation occurs.

(−)-*Thienylsuccinic acid* is obtained in a state of purity by recrystallisation from water of the crude acid liberated from the (−)-α-phenylethylamine salt. It resembles its optical antipode in all respects. Melting point 158°–162° (dec).

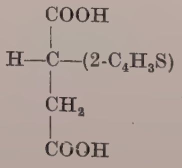
It is interesting to note the differences in stability between the optically active 2-thienylsuccinic and phenylsuccinic acids. WREN and WILLIAMS have studied the racemisation of phenylsuccinic acid under various conditions (12). They found that the acid was unaffected by prolonged action of potassium hydroxide solution at

100°, but readily underwent racemisation at 150°, depending rather upon the temperature than upon a specific action of the alkali hydroxide. This was unexpected, since it is known that optically active acids with a hydrogen atom at an asymmetric centre in the α-position, readily undergo alkali-catalysed racemisation (14). When the mother liquor from the first crystallisation of cinchonidine 2-thienylsuccinate was evaporated on a water bath, the liberated acid showed a considerably lower optical activity than was expected. A further investigation showed that the acid undergoes racemisation in boiling water and that the rate of racemisation increases in the presence of sodium hydroxide. This difference between phenylsuccinic and 2-thienylsuccinic acids is to be expected, and is probably due to the greater acidity of the thiophene compound. It would be interesting to study the behaviour of 3-thienylsuccinic acid in this respect.

Configuration of 2-thienylsuccinic acid. **Fredga** recently reported a method for steric correlation of aromatic and aliphatic compounds (15). The method includes fission of optically active 2-thiophene compounds to the corresponding *n*-butyl compounds with retention of the configurations. In the same way optically inactive and (+)-2-thienylsuccinic acids have now been transformed to racemic and (+)-*n*-butylsuccinic acids.

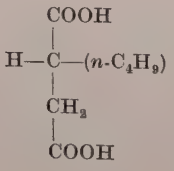


As (+)-2-thienylsuccinic acid gives (+)-*n*-butylsuccinic acid it is evident that 2-thienylsuccinic acids and *n*-butylsuccinic acids with the same direction of rotation have the same configuration. As **Matell** has shown the same to be valid for *n*-alkylsuccinic acids up to *n*-hexyl, and methylsuccinic acid has been connected to the glyceraldehyde system (17), the following formulas are obtained according to the convention of E. **Fischer**:



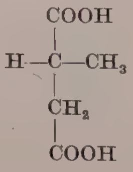
$$\begin{array}{c} COOH \\ | \\ H-C-(2\text{-}C_4H_3S) \\ | \\ CH_2 \\ | \\ COOH \end{array}$$

(+)



$$\begin{array}{c} COOH \\ | \\ H-C-(n\text{-}C_4H_9) \\ | \\ CH_2 \\ | \\ COOH \end{array}$$

(+)



$$\begin{array}{c} COOH \\ | \\ H-C-CH_3 \\ | \\ CH_2 \\ | \\ COOH \end{array}$$

(+)

The identification of the sulphur-free compound, obtained from (+)-2-thienylsuccinic acid showed some interesting features. The optical activity and equivalent weight were in accordance with those of (+)-*n*-butylsuccinic acid, and a mixture of the product and an authentic sample prepared by **Fredga** and **Sahlberg** (18), showed no melting-point depression. The melting point of the product was about four degrees too low, however, and could not be elevated in spite of several recrystallisations from different solvents. Furthermore, X-ray powder photographs of the product and of the authentic sample were quite different. But if the two samples were melted and then allowed to crystallise rapidly, the X-ray powder photographs were identical with each other and with that of the desulphurised product before melting. It must therefore be concluded that the product obtained is (+)-*n*-butylsuccinic acid, and that the two samples correspond to two modifications. MATELL has shown that the enanthiomorphs of *n*-butylsuccinic acid are dimorphic (16), but he has reported a melting point for the low melting modification which is appreciably lower than that obtained in this investigation. It is known, however, that polymorphy is very common in the succinic acid series. Thus racemic methylsuccinic acid has been shown to be polymorphic (19), with at least three modifications (20). A possible explanation of the anomalous melting point thus seems to be the existence of a third modification of (+)-*n*-butylsuccinic acid.

Experimental

2-Thienylsuccinic acid according to method 2:

Ethyl 2-thenalmalonate: 56.0 g (0.35 moles) of ethyl malonate, 38.1 g (0.34 moles) of 2-thiophenecarboxaldehyde, 5 ml of piperidine, 0.1 g of benzoic acid and 200 ml of benzene were boiled in a 1-l round-bottomed flask, fitted with a water separator connected to a reflux condenser. Heating was continued until water ceased to be formed. After cooling, the residue in the flask was washed twice with water, twice with 1-C hydrochloric acid and finally with a saturated solution of sodium hydrogen carbonate. The organic mixture was dried with anhydrous sodium sulphate, the benzene was removed by distillation and the residue fractionated *in vacuo*. Ethyl 2-thenalmalonate was collected at 150°–152°/1 mm Hg. Yield 72.2 g (84 %).

Ethyl β-(2-thienyl)-β-cyanopropionate: 18.0 g (0.07 moles) of ethyl 2-thenalmalonate and 300 ml of ethanol were placed in a 750 ml, three-necked, round-bottomed flask, fitted with a reflux condenser, mechanical stirrer, and dropping funnel. 9.3 g (0.14 moles) of potassium cyanide, dissolved in 10 ml of water, was added to the hot, stirred solution in the flask. Some potassium cyanide precipitated, but it soon dissolved and in a few minutes potassium hydrogen carbonate began to precipitate, and the solution darkened. The mixture was refluxed and stirred for 15 hours. The solution was filtered, the precipitate washed with ethanol, and the filtrate used directly for the preparation of 2-thienylsuccinic acid.

An improved yield would probably be obtained if the reaction time were reduced. The rapid formation of potassium hydrogen carbonate indicates that a reaction time of one or two hours would suffice.

2-Thienylsuccinic acid: To the dark solution of ethyl β-(2-thienyl)-β-cyanopropionate was added a solution of 3.0 g (0.08 moles) of sodium hydroxide in 10 ml of water. The mixture was refluxed for one hour. The ethanol was removed by distillation, during which some water was added from time to time to prevent evaporation to dryness. When all the ethanol was removed, the residue was made slightly acid with hydrochloric acid and refluxed for one hour. Decolourising charcoal was added and the hot solution of 2-thienylsuccinic acid was filtered and allowed to cool. The acid was extracted with ether, the ether was removed, and the acid recrystallised from water. Yield 8.0 g (57 %). M. p. 160°–162°.

2-Thienylsuccinic acid according to method 3.

Ethyl β-(2-thienyl)-α-cyanoacrylate: 15 g (0.13 moles) of 2-thiophenecarboxaldehyde, 17 g (0.15 moles) of ethyl cyanoacetate and 1 ml of piperidine were mixed in a 100 ml beaker. Heat was evolved, and the mixture assumed a dark red colour. After a few minutes it solidified completely. The product was dissolved in 100 ml of warm ethanol and allowed to cool. Orange-red needles of ethyl β-(2-thienyl)-α-cyanoacrylate soon precipitated. The needles were filtered off, washed with cold ethanol and dried in air. Weight 26 g (93 %). The crude product melted at 92°–95°. After several recrystallisations from ethanol it melted at 94°–98°.

118.7 mg of the product gave 7.06 ml of nitrogen of 751.0 mm pressure at a temperature of 21.0°.

$C_{10}H_9O_2NS$ N calc. 6.76 % found 6.85 %.

Ethyl β-(2-thienyl)-α, β-dicyanopropionate: 10 g (0.05 moles) of ethyl β-(2-thienyl)-α-cyanoacrylate was treated with 20 ml of dilute ethanol and 5 g (0.1 moles) of finely powdered sodium cyanide. The ester dissolved and the reaction was completed on heating on a steam bath for five minutes. (On heating for a longer time tarry products were obtained). The mixture was diluted with 100 ml of water and acidified with hydrochloric acid. A dark oil separated and was extracted with ether. The ether layer was used directly for the preparation of 2-thienylsuccinic acid.

2-Thienylsuccinic acid: The ether solution, containing ethyl β-(2-thienyl)-α, β-dicyanopropionate, was acidified with concentrated hydrochloric acid and allowed to stand for three days at room temperature, during which time a precipitate gradually separated.

The mixture was diluted with water, the ether layer separated, and the ether removed. The residue was dissolved in the water layer and the solution refluxed for two hours. After cooling it was extracted with ether, and the ether removed. The crude 2-thienylsuccinic acid was recrystallised from water. Weight 6 g (60 %). M. p. 160°–162°.

β-(2-Thienyl)-α-cyanoacrylic acid: A water solution of potassium cyanoacetate was prepared from chloroacetic acid, potassium carbonate and potassium cyanide, as described in Org. Syntheses *I*, 181 (1946). 12 ml of this solution, containing about 0.03 moles of potassium cyanoacetate, and 3.3 g (0.03 moles) of 2-thiophenecarboxaldehyde were heated on a water bath for half an hour. The aldehyde passed into solution, and after cooling the potassium salt of β-(2-thienyl)-α-cyanoacrylic acid was precipitated as a slightly yellow powder. The salt was dissolved in water, and the solution acidified with hydrochloric acid. The yellow crystals of the acid were filtered off and dried. Yield 3 g (50 %). The product decomposed at about 230°.

57.22 mg of acid, 9.50 ml of 0.03350–N NaOH
$C_8H_5O_2NS$. Equiv. wt. calc. 179.1, found 179.8.

Preliminary tests on resolution: 0.5 g (0.0025 moles) of optically inactive acid and 0.005 moles of the bases were dissolved in 15 ml of *ethanol*. The solutions were allowed to stand, and some water gradually added until crystals or oils were formed. The crystals were collected, the acid isolated and the optical activity measured in absolute ethanol.

Brucine in dilute ethanol: $[\alpha]_D^{25} = -34°$. The activity increases by about 10° with each recrystallisation.

Cinchonidine in dilute ethanol: $[\alpha]_D^{25} = +72°$.

Strychnine in dilute ethanol: $[\alpha]_D^{25} = +23°$.

Quinine in dilute ethanol gave a practically inactive acid.

Quinidine in dilute ethanol yielded a precipitate, which was shown to be the free alkaloid.

l-Ephedrine in dilute ethanol gave an oil.

0.2 g (0.001 moles) of the optically inactive acid and 0.002 moles of the bases were dissolved in 100 ml of *ethyl acetate* and a little *methanol*. The solutions were allowed to cool, and the precipitates were handled as described above.

(+)-*α-Phenylethylamine* yielded an acid with $[\alpha]_D^{25} = +48°$.

(+)-*Benzedrine* (21) yielded an acid with $[\alpha]_D^{25} = +37°$.

Resolution of 2-thienylsuccinic acid.

20 g (0.10 moles) of 2-thienylsuccinic acid and 60 g (0.20 moles) of cinchonidine were dissolved in 400 ml of hot ethanol. 400 ml of water was added and the solution left at room temperature for 24 hours. The precipitate was filtered off, washed with cold dilute ethanol, and dried. About 1 g of the salt was set apart, the acid isolated and the optical activity measured in absolute ethanol. The remaining salt was recrystallised from dilute alcohol, and the process repeated until the optical activity of the acid remained constant. The course of the resolution is seen below:

Crystallisation	1.	2.	3.
Ml of solvent	800	500	400
g salt obtained	41	30	25
$[\alpha]_D^{25}$ of acid	$+80°$	$+100°$	$+100°$

Cinchonidine (+)-*2-thienylsuccinate* crystallises in threadlike needles, which form large, hemispherical crystal aggregates. In air-dry condition it is free from crystal solvent.

165.2 mg of the product gave 10.10 ml of nitrogen of 750.0 mm Hg at a temperature of 20.8°.
$C_{46}H_{52}O_6N_4S$ N calc. 7.10 % found 7.06 %
73.21 mg in 10.00 ml of ethanol. $2\ \alpha_D^{25} = -1.083$, $[\alpha]_D^{25} = -73.9°$.

The mother liquor from the first crystallisation with cinchonidine was evaporated *in vacuo* at room temperature. The acid was liberated and 5.0 g was used for the isolation of the (−)-antipode. The acid, which showed an optical activity of about −60°, and 6.0 g of (−)-α-phenylethylamine were dissolved in a mixture of 300 ml of ethyl acetate and 50 ml of methanol, and left at room temperature for 24 hours. The crystals formed were filtered off and washed with cold ethyl acetate. The progress of the resolution was as described above.

Crystallisation	1.	2.	3.	4.	5.
ml of ethyl acetate	300	200	130	90	60
ml of ethanol	50	50	50	30	25
g salt obtained	6.5	4.2	3.0	2.0	1.2
$[\alpha]_D^{25}$ of acid	$-78°$	$-91°$	$-96°$	$-99°$	$-98°$

(−)-*α-Phenylethylamine* (−)-*2-thienylsuccinate* crystallises in small colourless needles. In air-dry condition it is free from crystal solvent.

114.2 mg of the product gave 6.26 ml of nitrogen of 750.0 mm Hg at a temperature of 20.2°.
$C_{24}H_{30}O_4N_2S$ N calc. 6.33 % found 6.35 %
16.01 mg in 10.00 ml of water $2\ \alpha_D^{25} = -0.628°$, $[\alpha]_D^{25} = -19.6°$.

(+)-2-Thienylsuccinic acid: 24 g of the cinchonidine salt were decomposed with 2-N sulphuric acid. The solution was extracted several times with ether, the ether layers then combined, and the ether removed. 5.5 g of crude acid was obtained and recrystallised several times from water. Sometimes the acid crystallised in transparent rods and sometimes as a colourless, granular powder. The acid was dried in air and in a desiccator over phosphorous pentoxide.

A transparent rod of the acid was placed in a capillary tube and heated. Between 125°–130° it lost its transparency without changing its shape. This clearly indicates a transformation to another modification, which has also been verified by X-ray powder analysis. The acid melts at 158°–162° (dec.). The comparatively large melting-point interval is not due to the presence of impurities, but to the thermal instability of the acid or to the existence of polymorphic modifications.

25.75 mg of acid (rods), 7.95 ml of 0.03215-N NaOH
44.95 mg of acid (powder), 6.46 ml of 0.06880-N NaOH
$C_8H_8O_4S$. Equiv. wt. calc. 100.0, found rods 100.7, powder 99.9
129.9 mg in 10.00 ml of ethanol. $2\ \alpha_D^{25} = +2.690°$, $[\alpha]_D^{25} = +103.5°$ $[M]_D^{25} = +207.0°$.

(−)-2-Thienylsuccinic acid: 1.2 g of (−)-α-phenylethylamine (−)-2-thienylsuccinate was decomposed with 2-N sulphuric acid. The solution was extracted several times with ether, the ether removed, and the acid recrystallised and dried as described for the (+)-acid, which it resembles in all respects.

26.11 mg of acid 8.12 ml of 0.03215-N NaOH
$C_8H_8O_4S$. Equiv. wt. calc. 100.0, found 100.0
100.3 mg in 10.00 ml of absolute ethanol. $2\ \alpha_D^{25} = -2.095°$
$[\alpha]_D^{25} = -103.9°$. $[M]_D^{25} = -207.8°$.

Racemisation of 2-thienylsuccinic acid: In order to remove the ethanol, the mother liquor from the first crystallisation of cinchonidine 2-thienylsuccinate was evaporated on a water bath to about ⅓ of its original volume. The acid liberated showed an optical activity of −10°, which was far less than expected. In a second run, the mother liquor was evaporated *in vacuo* at room temperature. The liberated acid now showed the expected optical activity of about −60°. Evidently racemisation occurred at higher temperatures. To test whether the racemisation was only a temperature effect or whether it was also influenced by hydroxyl ions, the following experiments were carried out:

a. 0.3350 g of pure (+)-thienylsuccinic acid was made up to 10.00 ml with 2-N sodium hydroxide. $[\alpha]_{5461}^{25} = +51°$. The solution was refluxed for two hours, after which it was so turbid that no reliable optical measurements could be performed. The solution was acidified, the acid extracted with ether, and the ether removed. The remaining acid was dried, and the optical activity measured in absolute ethanol.

0.1051 g in 10.00 ml of ethanol $2\ \alpha_D^{25} = +0.08°$, $[\alpha]_D^{25} = +3.9°$.

b. 0.2861 g of 2-thienylsuccinic acid, containing an excess of (+)-acid, was dissolved in water and made up to 25.0 ml. $2\ \alpha_D^{25} = +1.95°$, $[\alpha]_D^{25} = +85.2°$. The solution was refluxed for two hours, filtered, and the optical activity measured. $2\ \alpha_D^{25} = +1.48°$ $[\alpha]_D^{25} = +65.5°$.

The great difference in the rate of racemisation in the two experiments indicates that the rate depends on the concentration of hydroxyl ions. In this respect 2-thienylsuccinic acid differs from phenylsuccinic acid (12).

Solid (+)-2-thienylsuccinic acid was heated in a test tube at 140° for two hours. The X-ray powder photographs of the product before and after heating were quite different (13).

0.1195 g of the heated product in 10.00 ml of ethanol.

2 $\alpha_D^{25} = +2.45°$ $[\alpha]_D^{25} = +102.5°$.

(+)-2-Thienylsuccinic acid was heated at 160°–165° for one minute. 90.7 mg in 10.00 ml of absolute methanol 2 $\alpha_D^{25} = +0.70$ $[\alpha]_D^{25} = +38°$.

Conversion of 2-thienylsuccinic acids to n-butylsuccinic acids.

The Raney nickel catalyst was prepared as described by Vogel (22).

0.5 g (0.0025 moles) of optically inactive 2-thienylsuccinic acid and 2.5 g of potassium carbonate were dissolved in 500 ml of water. 10 g of Raney nickel suspension and 25 ml of ethanol were added and the mixture refluxed for two hours. The nickel was filtered off, the filtrate acidified with dilute sulphuric acid and extracted several times with ether. The ether layers were combined and the ether removed. The residue soon solidified. There was a strong smell of acetic acid, derived from oxidation of the ethanol. After several recrystallisations from hydrochloric acid 0.2 g of *n*-butylsuccinic acid remained. M. p. 81°–82°.

27.64 mg of acid 8.54 ml of 0.03700-N NaOH

$C_8H_{14}O_4$. Equiv. wt. calc. 87.1, found 87.5.

Mixed with an authentic sample (18), m.p. 81°–83°.

2 g (0.01 moles) of (+)-thienylsuccinic acid were dissolved in a mixture of 50 ml of 2-C sodium carbonate, 20 ml of ethanol and 200 ml of water. 40 g of Raney nickel catalyst were added, and the mixture refluxed for two hours. The nickel was filtered off, and the acid isolated as described above. 1.6 g of crude acid was obtained. After several crystallisations from hydrochloric acid and from a mixture of ether and petrol (b. p. 40°–60°) 1.0 g of acid, melting at 77°–79°, was obtained.

Mixed with an authentic sample (18), m.p. 77°–81°.

20.58 mg of acid 6.30 ml of 0.03738-N NaOH

$C_8H_{14}O_4$. Equiv. wt. calc. 87.1, found 87.4

13.02 mg of acid in 10.00 ml of ethanol. 2 $\alpha_D^{25} = +0.700$ $[\alpha]_D^{25} = +26.9°$.

Fredga and Sahlberg report for (+)-*n*-butylsuccinic acid (18) m.p. 82.5°–83.5°, $[\alpha]_D^{25} = +26.5°$.

Matell (16) reports for low melting *n*-butylsuccinic acid a melting point of 74°.

X-ray powder photographs were taken the results of which are mentioned in the theoretical part.

The author wishes to express his thanks to Professor Arne Fredga for valuable discussions and for the authentic samples of *n*-butylsuccinic acids used in the identification of the desulphurised products. The author is also indebted to Mr S. Gronowitz, who kindly placed some results at his disposal, and to Mr R. Öhberg for assistance with part of the experimental work.

Part of the expenses involved in this investigation were defrayed by a grant from the Nobel Fund of the Royal Swedish Academy of Science (Kemiska prisgruppens särskilda fond).

SUMMARY

Four general methods for the preparation of substituted succinic acids have been applied to the synthesis of 2-thienylsuccinic acid, which has also been resolved into optical antipodes.

The optically active 2-thienylsuccinic acids have been shown to exist in at least two modifications. The acids decompose at their melting points. They undergo racemisation in water solution at 100°, the rate of racemisation increasing in the presence of hydroxyl ions, which seems to be in contrast to the behaviour of the optically active phenylsuccinic acids.

Optically active 2-thienylsuccinic acid has the same configuration as the *n*-butylsuccinic acid with the same direction of rotation. As the configuration of these latter acids is known, 2-thienylsuccinic acid is thus related to the glyceraldehyde system.

Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala, October 1953.

REFERENCES

1. K. Pettersson, Acta Chem. Scand. *4*, 1319 (1950).
2. S. Wideqvist, Arkiv Kemi, Mineral. Geol. *26A* nr 16 (1948).
3. K. Pettersson, Acta Chem. Scand. In print.
4. J. Bredt and J. Kallen, Ann. *293*, 348 (1896).
5. S. Wideqvist, Arkiv Kemi *3*, 289 (1951).
6. ——, Arkiv Kemi, Mineral. Geol. *14B* nr 19 (1941).
7. S. Gronowitz, Private communication.
8. Org. Syntheses *31*, 108 (1951).
9. Chi-yi Hsing and Lien-tang Li, J. Am. Chem. Soc. *71*, 774 (1949).
10. K. Schaum, K. Schaeling and F. Klausing, Ann *411*, 193 (1916).
11. A. Fredga and M. Matell, Bull. Soc. Chim. Belg. *62*, 47 (1953).
12. H. Wren and H. Williams, J. Chem. Soc. *109*, 572 (1916).
13. K. Pettersson, To be published.
14. A. McKenzie and S. T. Widdows, J. Chem. Soc. *107*, 702 (1915).
15. A. Fredga, Arkiv Kemi *6*, 277 (1953).
16. M. Matell, Arkiv Kemi *5*, 17 (1952).
17. A. Fredga and E. Leskinen, Arkiv Kemi, Mineral. Geol. *19B* nr 1 (1944).
18. A. Fredga and U. Sahlberg, Arkiv Kemi, Mineral. Geol. *18A* nr 16 (1944).
19. A. Fredga, Arkiv Kemi, Mineral. Geol. *15B* nr 23 (1942).
20. G. D. Buckley, J. Chem. Soc. *1953*, 1325.
21. M. Matell, Acta Chem. Scand. *7*, 698 (1953).
22. A. Vogel, Practical Organic Chemistry (1948) p. 807.

Tryckt den 4 mars 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 6

Communicated 11 November 1953 by A. WESTGREN and G. HÄGG

The structure of the hexatantalate ion in $4\,K_2O \cdot 3\,Ta_2O_5 \cdot 16\,H_2O$

By INGVAR LINDQVIST and BERTIL ARONSSON

With 1 figure in the text

In a previous paper (1) it has been proved that the ion $Nb_6O_{19}^{8-}$ exists in the salt $2\,Na_7HNb_6O_{19} \cdot 31\,H_2O$ ($7\,Na_2O \cdot 6\,Nb_2O_5 \cdot 32\,H_2O$). The formula of this ion immediately suggests that the ion $Ta_6O_{19}^{8-}$ should exist in tantalates of the type $4\,K_2O \cdot 3\,Ta_2O_5 \cdot aq.$

Salts of this type were first prepared by MARIGNAC (2), who investigated the salt $4\,K_2O \cdot 3\,Ta_2O_5 \cdot 16\,H_2O$ crystallographically. The mole ratio 4 : 3 was criticized by JANDER (3), who obtained the ratio 7 : 5. This formula was also in better agreement with his diffusion measurements, which indicated the existence of pentatantalate ions in aqueous solution. The analytical discrepancy was explained by WINDMAISSER (4), who found a mole ratio varying between 4 : 3 and 7 : 5. A subsequent preliminary X-ray study (5) showed, however, that the number of Ta atoms in the unit cell is always a multiple of 6 and not of 5. The variation in composition was explained as a replacement of H_2O by KOH within certain limits (5). Another explanation was given by ROBINSON (6), who considered the effect to be due to adsorption.

Crystals

Crystals were prepared according to the method of WINDMAISSER (4) by melting Ta_2O_5 with KOH in a silver crucible. The crystal mass formed was dissolved in water at room temperature, and the solution left to crystallize over H_2SO_4 in vacuum. The crystals obtained were analyzed by Dr. F. NYDAHL, whom we wish to thank for his valuable aid. The mole ratio $K_2O : Ta_2O_5 : H_2O$ was found to be 1.33 : 1.00 : 5.26, in good agreement with 1.33 : 1.00 : 5.33 calculated for $4\,K_2O : 3\,Ta_2O_5 : 16\,H_2O$. The cell dimensions of the monoclinic crystals were determined from powder photographs, and found to be $a = 11.91$ Å, $b = 16.65$ Å, $c = 18.46$ Å, $\beta = 95^{\circ}.7$ (HALLA (5) found $a = 12.08$ Å, $b = 16.87$ Å, $c = 18.37$ Å and $\beta = 95^{\circ}.23$). The number of Ta atoms in the unit cell is 24 according to HALLA (5). A roughly needle-shaped crystal (~ 0.07 mm thick), quickly dried between filter papers and covered by a layer of paraffin (5), was used for the single crystal work. Weissenberg photographs were taken around the c axis of the crystal (layer lines 0–8). The reflections $h\,k\,l$ exist only for $h + k + l = 2\,n$ and $h\,0\,l$ only for $h = 2\,n$ and $l = 2\,n$, $0\,k\,0$ only for $k = 2\,n$. The most symmetrical space-group possible is $I\,2/c$ (or $I\,2/a$). (HALLA gives the space-group $C\,2/c$, probably due to some mistake.) We have chosen the notation $I\,2/c$. Relative $|F|^2$ values were calculated from the estimated intensities, using LU's curves (7).

Tantalum positions

The general position of the space-group $I\,2/c$ is (8 *f*):

$$(x,\ y,\ z)\quad (\bar{x},\ \bar{y},\ \bar{z})\quad (\bar{x},\ y,\ \tfrac{1}{2}-z)\quad (x,\ \bar{y},\ \tfrac{1}{2}+z)$$
$$(x+\tfrac{1}{2},\ y+\tfrac{1}{2},\ z+\tfrac{1}{2})\quad (\tfrac{1}{2}-x,\ \tfrac{1}{2}-y,\ \tfrac{1}{2}-z)\quad (\tfrac{1}{2}-x,\ \tfrac{1}{2}+y,\ \bar{z})$$
$$(\tfrac{1}{2}+x,\ \tfrac{1}{2}-y,\ z).$$

A complete three-dimensional Patterson synthesis was carried out, using the Hägg-Laurent machine (8). By a logical analysis of these $P(x, y, z)$ functions (cf. (1)), it was possible to derive the following three Ta positions:

8 Ta_{I} in 8 (*f*): $x_{I} = 0.11$ $y_{I} = 0.07$ $z_{I} = \overline{0.08}$
8 Ta_{II} in 8 (*f*): $x_{II} = \overline{0.07}$ $y_{II} = 0.12$ $z_{II} = 0.04$
8 Ta_{III} in 8 (*f*): $x_{III} = 0.15$ $y_{III} = 0.00$ $z_{III} = 0.09$

These atomic positions would give rise to the following maxima in $P(x, y, z)$. (Only the region $x = 0 - \frac{1}{2}$, $y = 0 - \frac{1}{4}$, $z = 0 - 1$ has been considered, because of the symmetry of the space-group):

Ta_{I}–Ta_{I}	$(0.22,\ 0.14,\ \overline{0.16})$	(170)	**1**
	$(0.22,\ 0.00,\ 0.34)$	(350)	**2**
	$(0.00,\ 0.14,\ 0.50)$	(290)	**2**
Ta_{II}–Ta_{II}	$(0.14,\ 0.24,\ \overline{0.08})$	(160)	**1**
	$(0.14,\ 0.00,\ 0.42)$	(335)	**2**
	$(0.00,\ 0.24,\ 0.50)$	(300)	**2**
Ta_{III}–Ta_{III}	$(0.30,\ 0.00,\ 0.18)$	(300)	**2**
	$(0.30,\ 0.00,\ \overline{0.32})$	(290)	**2**
	$(0.00,\ 0.00,\ 0.50)$	(810)	**4**
Ta_{I}–Ta_{II}	$(0.18,\ 0.05,\ \overline{0.12})$	(360)	**2**
	$(0.04,\ 0.19,\ \overline{0.04})$	(310)	**2**
	$(0.04,\ 0.05,\ 0.46)$	(320)	**2**
	$(0.18,\ 0.19,\ 0.38)$	(320)	**2**
Ta_{I}–Ta_{III}	$(0.04,\ 0.07,\ 0.17)$	(380)	**2**
	$(0.26,\ 0.07,\ 0.01)$	(360)	**2**
	$(0.26,\ 0.07,\ 0.51)$	(320)	**2**
	$(0.04,\ 0.07,\ \overline{0.33})$	(320)	**2**
Ta_{II}–Ta_{III}	$(0.22,\ 0.12,\ 0.05)$	(360)	**2**
	$(0.08,\ 0.12,\ 0.13)$	(320)	**2**
	$(0.08,\ 0.12,\ \overline{0.37})$	(360)	**2**
	$(0.22,\ 0.12,\ 0.55)$	(330)	**2**

These maxima are all found in $P(x, y, z)$, and their relative magnitudes are given in the third column compared with the weight (fourth column) of the vectors calculated from the space-group symmetry. The agreement is good. There are no other maxima in $P(x, y, z)$ of as large a magnitude as the above, but there are a few slightly lower ones.

Oxygen configuration of the complex ion

The interatomic distances Ta–Ta for the six Ta atoms $\pm(0.11, 0.07, 0.08)$, $\pm(\bar{0}.07, 0.12, 0.04)$ and $\pm(0.15, 0.00, 0.09)$ (Table 1) are in good agreement with the results obtained in $Nb_6O_{19}^{8-}$ (1). The idealized model of $Ta_6O_{19}^{8-}$ (Fig. 1) would

Table 1. The shortest Ta–Ta distances in Å

	a	b	c	d	e	f
a	—	4.6	3.3	3.3	3.3	3.3
b	4.6	—	3.3	3.3	3.3	3.3
c	3.3	3.3	—	3.3	4.4	3.3
d	3.3	3.3	3.3	—	3.3	4.7
e	3.3	3.3	4.4	3.3	—	3.3
f	3.3	3.3	3.3	4.7	3.3	—

The notation *a–f* is in accordance with Fig. 1.

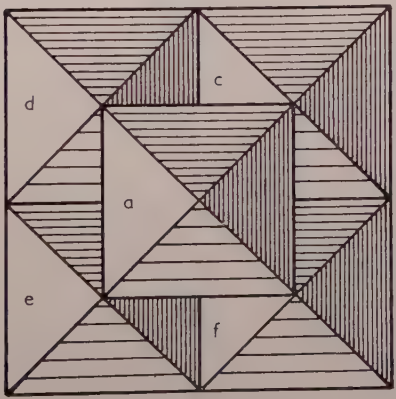


Fig. 1. Idealized structure of $Ta_6O_{19}^{8-}$. The octahedron *b* below *a* cannot be seen. Ta atoms in the centers of the octahedra and oxygen atoms at the corners.

also give rise to Ta–O maxima at $\pm(0.11, 0.07, \overline{0.08})$, $\pm(\overline{0.07}, 0.12, 0.04)$ and $\pm(0.15, 0.00, 0.09)$. Rather extended maxima are actually found near these positions.

No attempt has been made to determine the K^+ and H_2O positions from the X-ray data.

Discussion

The ion $Ta_6O_{19}^{8-}$ is directly related to the analytical formula $4\,K_2O \cdot 3\,Ta_2O_5 \cdot 16\,H_2O$, which should be written $K_8Ta_6O_{19} \cdot 16\,H_2O$ (cf. $2\,Na_7HNb_6O_{19} \cdot 31\,H_2O$). Of course, it would be necessary to carry out a more extensive investigation, with a more thorough discussion, before it could be stated definitely whether the ion $Ta_6O_{19}^{8-}$ also exists in aqueous solution (cf. $Mo_7O_{24}^{6-}$ and $Mo_8O_{26}^{4-}$, which have been discussed more completely (7, 8)). JANDER's theory about the existence of a pentatantalate ion (3) is probably incorrect, however. The molecular weight found by him is actually about 20 % too high compared with that calculated for a $Ta_5O_{16}^{7-}$ ion, and therefore in better accord with $Ta_6O_{19}^{8-}$. The variation in composition mentioned in the introduction cannot be explained without knowledge of the complete structure.

SUMMARY

The existence of the ion $Ta_6O_{19}^{8-}$ (Fig. 1) in the salt $4\,K_2O \cdot 3\,Ta_2O_5 \cdot 16\,H_2O$ has been proved by a structure determination. The salt should therefore be written $K_8Ta_6O_{19} \cdot 16\,H_2O$.

The Swedish Natural Science Research Council supported the work financially, and this is gratefully acknowledged. We also wish to thank Professor G. HÄGG for the facilities he has put at our disposal.

Institute of Chemistry, University of Uppsala, Uppsala, Sweden.

REFERENCES

1. LINDQVIST, I., Arkiv Kemi *5*, 247. 1953.
2. MARIGNAC, J. C. G., Ann. Chim. Phys. [4] *9*, 249. 1866.
3. JANDER, G. and SCHULTZ, H., Z. anorg. Chem. *144*, 225. 1925.
4. WINDMAISSER, F., Z. anorg. Chem. *248*, 283. 1941.
5. HALLA, F., NETH, A. and WINDMAISSER, F., Z. Krist. *104*, 161. 1942.
6. ROBINSON, H. T. S. and BRITTON, R. A., J. Chem. Soc. 2265, 1932.
7. LU, CHIA-SI, Rev. Sci. Instr. *14*, 331. 1943.
8. HÄGG, G. and LAURENT, T., J. Sci. Instr. *23*, 155. 1946.
9. LINDQVIST, I., Nova Acta Reg. Soc. Sci. Ups. *IV 15*, N:o 1. 1950.
10. —— Acta Chem. Scand. *5*, 568. 1951.

Tryckt den 1 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 7

Read 28 October 1953

Starch degradation by salivary amylase II

By Karl Myrbäck

With 3 figures in the text

In the first paper of this series (1) the end-products of starch degradation by salivary amylase were found to be: maltose (about 73 per cent of the theoretical value), glucose (about 12 per cent), and residual dextrins (about 15 per cent). In confirmation of earlier investigations from this laboratory, the residual dextrins were found to be practically stable, *i.e.* they were not attacked by salivary amylase to any measurable extent within reasonable time. The average degree of polymerization of the residual dextrins was at least four units. Contrary to repeated statements by K. H. Meyer *et al.* (2) no isomaltose was found among the non-fermentable products.

In a subsequent paper (3) experiments were reported in which the (more or less) stable dextrins of salivary amylase were isolated, roughly fractionated by alcohol precipitation, and the occurrence in the fractions of saccharides of varying degree of polymerization was investigated by means of paper chromatography. Saccharides with 4, 5, 6, 7 and probably 8 units were shown to be present in most fractions. When such fractions were treated for a very long time with very large amounts of salivary amylase a very slow and incomplete hydrolysis was observed. By paper chromatography it was found that this hydrolysis affects the residual dextrins with more than four units. The amount of tetrasaccharide was increased in a way that seems to warrant the assumption of tetrasaccharides as the true limit dextrins of salivary amylase. If this result can be verified it seems to be of considerable interest to determine the structure of the tetrasaccharide(s), *i.e.* the nature of the glucosidic linkages therein. Some new experiments are reported in the following.

Experimental

About 850 g of potato starch (dry weight) was boiled with 15 liter of water. The paste was cooled to 45°. 175 ml filtered saliva and some toluene was added. The pH was brought to 6.7 with a few drops of NaOH. The mixture was kept at 45° and the saccharification was estimated by sugar determinations according to the Willstätter-Schudel and Bertrand methods: Table 1. The mixture was cleared in a Sharpless supercentrifuge after 20 days, and 13 l fluid was recovered. Fermentation experiments showed the presence of about 11 per cent glucose, about 70 per cent maltose and about 18 per cent residual, non-fermentable dextrin.

Paper chromatograms were prepared according to the method described earlier (4). The maltose and glucose spots were very strong; weaker spots showed the presence of maltotriose and dextrins with 4–7 units (No. 1 in Fig. 1).

Table 1.

Time	Percent hydrolysis
2 days	38
9 ,,	45
14 ,,	48
20 ,,	49

Table 2.

Time	Percent "glucose"
0	32.2
2 hrs	34.8
8 days	46.4
13 ,,	47.8
17 ,,	51.9
24 ,,	51.5

The volume was reduced to 7 liters and practically all fermentable sugar was removed by treatment with baker's yeast. After filtration through kieselguhr the alcohol was destilled off and the volume reduced to 1 liter. This solution contained about 180 g dextrin and the average degree of polymerization of the dextrins was 5.7. The chromatogram (No. 2 in Fig. 1) showed a very weak spot corresponding to maltotriose (which is fermented rather slowly by the yeast used) and stronger dextrin spots. Limit dextrins with 4–7 units were represented; the strongest spot corresponds to the hexasaccharide IM-6 (4).

800 ml of the solution, containing about 144 g dextrin was mixed with 150 ml filtered saliva and the mixture was kept under toluene at 45°. The increase in reducing groups was determined and calculated as mg apparent glucose/ml (Table 2). The hydrolysis obviously came to a standstill after some 17 days. It shall not be argued that the conditions reached at that time represent an absolute endpoint of the amylatic reaction, but the products must at least be regarded as practically stable. Fermentation experiments with baker's yeast revealed the presence of 8.0 per cent glucose and 17.7 per cent maltose (possibly including some maltotriose). The chromatograms (one is shown as No. 3 in Fig. 1) showed strong glucose, maltose and dextrin spots. Dextrins with 4–7 units are present and a weak spot with R_G = about 0.02 indicates the presence of some still higher dextrin ("spot X", cf. (4)). After fermentation the yeast was removed by filtration and the filtrate was reduced to 310 ml. The chromatograms showed traces of fermentable sugars (maltose and maltotriose) and the presence of dextrins with 4–7 units together with small amounts of a higher dextrin (No. 4 in Fig. 1).

300 ml of the solution was precipitated with absolute alcohol:

400 ml alcohol gave 36.4 g dextrin fraction S I (dry weight)
500 ml ,, ,, 16.8 g ,, ,, S II
650 ml ,, ,, 11.1 g ,, ,, S III
950 ml ,, ,, 7.7 g ,, ,, S IV
1950 ml ,, ,, 5.3 g ,, ,, S V

The filtrate from fraction S V was reduced to 30 ml and precipitated with 250 ml absolute alcohol. 2.7 g dextrin fraction S VI was recovered. The filtrate was evaporated to a thick sirup and taken up in 25 ml water. This fraction, called fraction S VII, contained 0.9 g substance, chiefly fermentable sugars (No. 11 in Fig. 1). Fraction S VI contained about 10 per cent slowly fermentable material, but the fractions S I–S V were practically non-fermentable. The total yield of residual-dextrin was 80.9 g, corresponding to some 14 per cent of the starch.

The dextrin fractions were investigated by paper chromatography. The conventional method for descending chromatography was used; details in (4). The various saccharides were characterized by their R_G values, *i.e.* by their paper chromatographic mobility, referred to that of glucose as a standard. The R_G value of glucose was determined in separate experiments and found to be 0.35.

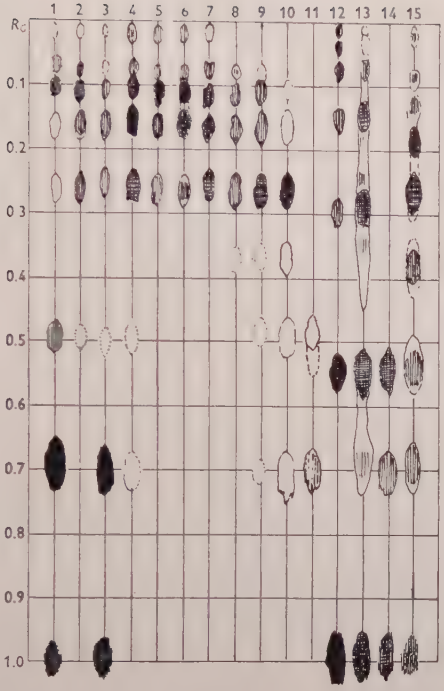


Fig. 1.

Discussion

From the chromatograms (Nos. 5–11 in Fig. 1) it is obvious that the fractions S I–S III contain limit dextrins with 4, 5, 6, and 7 units together with the higher dextrin(s) corresponding to "spot X". Fraction S IV contains tetra-, penta-, hexa-, and heptasaccharides, but "spot X" is absent. The hexasaccharides are abundant in all fractions S I–S IV. The fraction S V contains tetra-, penta-, and hexasaccharides only, and fraction S VI practically exclusively tetrasaccharides, contaminated by small amounts of a dextrin with three units, maltotriose and maltose.

These results agree completely with other findings from this laboratory: The limit dextrins with 4–7 units are extremely stable against salivary amylase, and the tetrasaccharide is the most stable among them.

The R_G-values of oligosaccharides with only 1,4-α-glucosidic linkages (maltose series) have been determined earlier (4), and the values are recorded in Table 3. These values were determined in experiments with hydrolysates of Schardinger's β-dextrin (cycloheptaamylose), containing the first seven members of the series. As a comparison the corresponding values for oligosaccharides containing only 1,6-α-glucosidic linkages (isomaltose series) have been determined. Material containing the lower members of this series was obtained in two different ways:

a) by incomplete hydrolysis of dextran with sulphuric acid at 100°. The acid was removed with baryum carbonate (No. 12 in Fig. 1).

b) by "reversion" of glucose in concentrated hydrochloric acid at +2°. Several fractions of the products were obtained by alcohol precipitation. The fractions were

Table 3. R_G and q values of oligosaccharides; $q = \log\,(1/R_F - 1)$.

n	Maltose series		Isomaltose series		Limit dextrin series	
	R_G	q	R_G	q	R_G	q
1	1	0.270	–	–	–	–
2	0.704	0.484	0.550	0.624	0.550	0.624
3	0.502	0.670	0.290	0.987	0.368	0.829
4	0.333	0.878	0.143	1.279	0.264	0.992
5	0.210	1.100	0.070	1.600	0.171	1.197
6	0.138	1.295	–	–	0.112	1.390
7	0.093	1.474	–	–	0.070	1.600

washed free from acid by alcohol and ether (No. 13 in Fig. 1; No. 14 is a control: glucose, maltose and isomaltose).

It was found, that the spots on the paper chromatograms for both preparations coincided completely. In the chromatograms of the reversion products, weak spots could be seen between the strong spots belonging to the isomaltose series. This question will be discussed in a forthcoming paper. The R_G-values found for the 1,6-oligosaccharides are listed in Table 3.

When salivary amylase limit dextrins, such as the preparations mentioned above, are hydrolyzed incompletely with acid and the hydrolysates are tested by paper chromatography, spots corresponding to the following substances are observed: 1) glucose, 2) maltose, 3) sometimes maltotriose showing that in some limit dextrin molecules there are at least two adjacent 1,4-linkages, 4) isomaltose showing the presence of at least one 1,6-linkage, 5) panose, 6) a series of spots coinciding with the spots given by the original limit dextrin preparation (No. 15 in Fig. 1). Our present knowledge of the constitution of starch (amylopectin) allows the conclusion that the majority of, or perhaps all, amylase limit dextrins are parts of the ramified substrate molecules and originate from the ramification points (= 1,6-linkages). As pointed out in several papers from this laboratory (5, 6), it then seems permissible to assume that all or nearly all limit dextrin molecules contain *one* isomaltose linkage together with a number of maltose linkages. (The limit dextrin(s) corresponding to "spot X" in the chromatograms may perhaps contain more than one isomaltose linkage). In the limit dextrins and in their hydrolysates, we therefore have the following saccharides, beside fermentable sugars:

One 1,6-linkage = isomaltose
one 1,6-linkage + one maltose linkage = panose (or isomer)
one 1,6-linkage + two maltose linkages = tetrasaccharides
one 1,6-linkage + three maltose linkages = pentasaccharides
etc.

Dextrins of this kind obviously are more or less resistant to the action of salivary amylase. The stability increases with decreasing chain length, so that the tetrasaccharide(s) seem to be practically if not completely stable (3).

There is a clear correlation between structure and molecular size of saccharides and their mobility in paper chromatography (7). For instance, FRENCH *et al.* have shown that the saccharides of the maltose (amylose) series, *i.e.* those containing 1,4-linkages

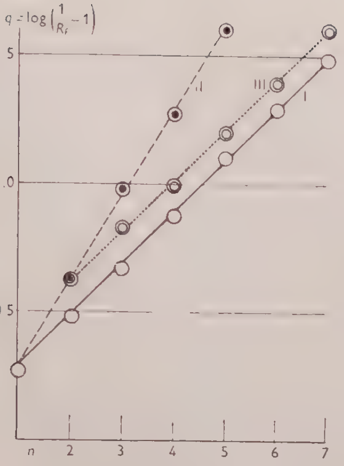


Fig. 2.

exclusively, fall into a regular series. If log R_F is plotted against n (number of glucose units in the saccharide) a practically straight line is obtained, which is quite in accordance with results from this laboratory (4). Better understood theoretically is the straight line relation between n and log $(1/R_F - 1)$ observed by JEANES *et al.* (7) and others. In Fig. 2 this function is used and the full-drawn line I represents the oligosaccharides of the maltose series according to our experiments. The dashed line II corresponds to the series of saccharides with 1,6-linkages exclusively (isomaltose, isomaltotriose etc.). When these lines are extrapolated to $n = 1$ (glucose) they meet on the ordinate and approximately at the value of log $(1/R_F - 1)$ found for glucose. If the corresponding values for the limit dextrins and their hydrolysis products are plotted in the same way we get a third straight line III, dotted in Fig. 2, starting from the isomaltose value and parallel to line I (maltose series).

As pointed out by FRENCH and WILD (8) it appears possible to make the general statement that increasing the size of a saccharide by one glucose unit will decrease the papergram mobility by an amount which depends on the type of hexose unit being added and on its mode of attachment. Our results are in good agreement with this assumption. For instance, for every step in the maltose series the value of $q = \log (1/R_F - 1)$ increases by about 0.21, intimating that this value corresponds to a 1,4-α-glucosidic linkage. Similarly a value of $q = 0.36$ would correspond to the 1,6-α-glucosidic linkage.

The q values of our limit dextrin series can obviously be calculated approximately from the formula

$$q = q_{\text{Glucose}} + 0.36 + 0.21\,(n - 2)$$

or

$$q = q_{\text{Isomaltose}} + 0.21\,(n - 2).$$

This would mean that the members of the limit dextrin series are oligosaccharides derived from isomaltose by successive addition of one glucose unit attached by a 1,4-α-glucosidic linkage. Obviously this conclusion is identical with our old working hypothesis that the stable detrins are oligosaccharides containing one 1,6-linkage together with a certain number of 1,4-linkages.

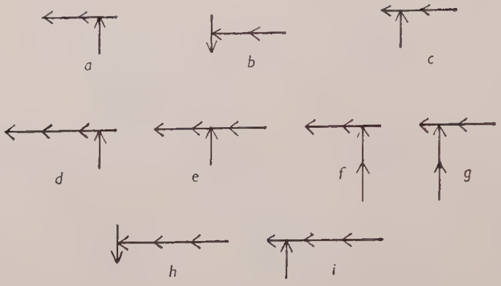


Fig. 3.

It is tempting to ask if the determination of the papergram mobilities might also allow the determination of the position of the isomaltose linkage in a limit dextrin molecule. Already in the trisaccharide three isomers are possible: 4-α-isomaltosido-glucose (panose), 6-α-maltosido-glucose and 4,6-bis-α-glucosido-glucose (Formulas $a - c$ in Fig. 3). The presence of one maltose and one isomaltose linkage in the trisaccharide fraction of a limit dextrin preparation was shown by MYRBÄCK and AHLBORG in 1940 (9), using the classical methylation method. It seems safe by now, among other things because of the coincidence of the papergram spots, to assume that the limit dextrin trisaccharide is panose.

The *q*-value of the limit dextrin trisaccharide is 0.64 and those of maltotriose and isomaltotriose are 0.47 and 0.77, respectively. Since no other spots could be detected between these values it appears safe to assume that the trisaccharide is panose and that the isomerides, if present at all, can occur only in very small amounts.

The limit dextrin trisaccharide(s) may originate from one or more of the tetrasaccharides depicted schematically in Fig. 3: *d–i* by liberation of glucose (or from pentasaccharides by liberation of maltose). In our chromatograms of limit dextrins and their hydrolysates, the tetrasaccharide spot with $R_G = 0.264$ is fairly well defined, just about as well as well as the spots corresponding to well-known homogeneous sugars. Thus we have to conclude either that the limit dextrin tetrasaccharide fraction contains *one* of the possible tetrasaccharides (Fig. 3) as its main constituent possibly together with quite small amounts of the isomerides, or that the isomerides have practically the same R_G-values when butanol-pyridine-water 6 : 4 : 3 is used for irrigation of the papergrams. Further investigations of other limit dextrin fractions, using different irrigation fluids are necessary to settle this point. On the other hand it may well be pointed out, that maltotetraose has an R_G-value of 0.333 and isomaltotetraose, *i.e.* the tetrasaccharide with three isomaltose linkages, has $R_G = 0.143$. When comparing these values with the value $R_G = 0.264$ for the limit dextrin tetrasaccharide it hardly seems possible that all tetrasaccharides visualized in Fig. 3: *d–g* should have practically the same R_G-values.

It should be pointed out also that the occurrence of all possible tetrasaccharides (Fig. 3: *d–i*) in the tetrasaccharide limit dextrin fraction is most unlikely in itself. For instance, if the tetrasaccharides *h* and *i* are formed at all, which seems extremely doubtful, they should not be stable since maltose can be split off from the non-reducing end-chains. Furthermore, since panose (Fig. 3: *a*) is the only trisaccharide or at least the main product of the trisaccharide fraction, it must be concluded that the tetrasaccharide *g* can not occur to any considerable extent since panose can not be formed from it. In the acid hydrolysis (and in the enzymatic hydrolysis which may be brought about by certain α-amylases, *e.g.* the malt enzyme) panose can be formed only from the tetrasaccharides *d–f*. On acid hydrolysis *d* must yield panose, maltose, isomaltose, and glucose as the main products. Maltotriose is also possible as a hydrolysis product but, since the 1,6-linkage is split at a considerably lower rate than are the 1,4-linkages, the yield of maltose must be very low.

The tetrasaccharide *e* must yield panose, (maltotriose), trisaccharide *c*, isomaltose and glucose. Finally the tetrasaccharide *f* should yield panose, trisaccharide *b*, (maltose), isomaltose and glucose. Obviously *d* is the only tetrasaccharide which would, on acid hydrolysis, yield panose (and maltotriose) without admixture of the trisaccharides *b* or *c*. As mentioned before, it seems rather unlikely that *a*, *b*, and *c* would have the same R_G-values. Thus the structure of the stable tetrasaccharide, produced from amylopectin by salivary amylase, appears to be, as far as can be judged from experiments reported here, that of tetrasaccharide *d*, 4-α-panosido-glucose.

SUMMARY

The residual dextrins, stable or practically stable against salivary amylase, contain 4–7 glucose units; small amounts of higher dextrins are present. The papergram mobilities of the stable dextrins have been determined. If the function $q = \log (1/R_F - 1)$ is plotted against molecular size a straight line relationship is found. The line starts from the value corresponding to isomaltose and is parallel to the line representing the oligosaccharides of the maltose (amylose) series. This result agrees completely with the theory of the structure of the limit dextrins proposed by the author long ago.

The stability of the limit dextrins increases with decreasing number of glucose units in the molecule. The tetrasaccharide seems to be quite stable. Evidence is presented indicating that this saccharide is 4-α-panosido-glucose.

The experimental part of this investigation has been carried out by Mrs. E. WILLSTAEDT whose assistance is gratefully acknowledged.

Institute of Organic Chemistry and Biochemistry, University, Stockholm.

REFERENCES

1. MYRBÄCK, K. and SVANBORG, K., Arkiv Kemi *5*, 317 (1953).
2. MEYER, K. H., FULD, M., and BERNFELD, R., Experientia *3*, 411 (1947); MEYER, K. H., and GONON, W. F., Helv. Chim. Acta *34*, 308 (1951).
3. MYRBÄCK, K. and WILLSTAEDT, E., Arkiv Kemi *6*, 426 (1953).
4. ——, Arkiv Kemi *6*, 417 (1953).
5. MYRBÄCK, K. *et al.*, Biochem. Z. *307*, 53, 67 (1940); *308*, 187 (1941); *311*, 213, 227, 234, 242 (1942); J. prakt. Chem. N. F. *162*, 29 (1943); Svensk kem. tidskr. *58*, 119 (1946); Actualités biochimiques *10*, 12 (1947); Advances in carbohydrate chem. *3*, 251 (1948); Ergeb. Enzymforsch. *12*, 1 (1951).
6. MYRBÄCK, K. and NEUMÜLLER, G., In SUMNER, J. B. and MYRBÄCK, K., The Enzymes, Acad. Press, New York 1950. Vol. I, p. 653.
7. FRENCH, D. and KNAPP, D. W., J. Biol. Chem. *187*, 463 (1950); FRENCH, D., KNAPP, D. W., and PAZUR, J. H., J. Am. Chem. Soc. *72*, 5150 (1950); FRENCH, D., Science *113*, 352 (1951); JEANES, A., WISE, C. S., and DIMLER, R. J., Anal. Chem. *23*, 415 (1951).
8. FRENCH, D. and WILD, G. M., J. Am. Chem. Soc. *75*, 2612 (1953).
9. MYRBÄCK, K. and AHLBORG, K., Biochem. Z. *307*, 69 (1940).

Tryckt den 1 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 8

Communicated 2 December 1953 by ARNE FREDGA

An investigation of the mechanism for the formation of S-*tert.* alkyl thiuronium picrates

By LENNART SCHOTTE and ULLA BERGLUND-LARSSON

Recently two investigations of the preparation of S-alkyl-thiuronium picrates were published. (L. S. (1) and (2).) A possible explanation of the reaction mechanism was proposed and special interest was devoted to the study of the tertiary alkyl halides, which give anomalous results (3) in reaction with thiourea in ethanolic and methanolic solutions. Instead of the S-*tert.* alkyl thiuronium picrates the S-ethyl or methyl thiuronium derivatives are formed, when the reaction mixture is treated with picric acid. In the above mentioned publications it was shown possible to prepare the tertiary alkyl derivatives with either the corresponding tertiary alcohol or water as a solvent. This seems to be in good agreement with the proposed mechanism given in (1).

It is well known that tertiary alkyl halides usually react according to an S_1-mechanism, *i.e.* a first order substitution reaction, and in this case the reaction takes place with the solvent. If then the solvent is ethyl or methyl alcohol, the corresponding halides and the tertiary alcohol are formed. The primary alkyl compound, thus obtained, then reacts with thiourea, yielding the primary alkyl thiuronium derivative, probably in an S_2-reaction. If, however, the solvent is the corresponding tertiary alcohol, the S_1-reaction with the solvent is prevented or only leads to an exchange of radicals of the same kind, why the wanted tertiary thiuronium compounds are obtained without any difficulties. In order to ascertain whether this supposed exchange of radicals really takes place, the reaction was performed in a solution of tertiary alcohol, in this investigation *tert.* amyl alcohol, with tracer amounts of tritium in the alkyl group. If tritium was recovered in the S-*tert.* alkyl thiuronium picrate there must have been an exchange of radicals between the tertiary halide and the alcohol. This was also the case, thus elucidating the whole mechanism.

It is a necessary condition in measurements of this kind to control that no spontaneous hydrogen exchange takes place. For this reason some tritium water was added to the ordinary tertiary alcohol which was used as a solvent for the tertiary alkyl halide in the control reaction with thiourea. Some tritium was found in the thiuronium picrate, which indicates that a spontaneous hydrogen exchange occurs to a small extent. The exchange of radicals, however, is much more rapid than is the spontaneous hydrogen exchange.

Experimental

Measurement of tritium. The measurements of tritium were carried out with an apparatus described by MELANDER (4), (5). All organic compounds were burned and the water reduced to hydrogen, which was used for inside counting in a GEIGER-MÜLLER tube. The accuracy of the determinations is within ± 2 %. The unit of the specific activity used throughout the investigation refers to the GEIGER-MÜLLER tube employed and corresponds to about 250 disintegrations per millimole of hydrogen per minute. The corrections for the natural decay of tritium were made assuming its half-life to be 12.5 years.

The organic compounds, containing no sulphur or nitrogen, were burned in an oxygen atmosphere over red hot cupric oxide. When S-*tert.* amyl thiuronium picrates were burned the oxygen was replaced by carbon dioxide and two further tubes were added behind the tube containing cupric oxide, the first of these tubes contained heated silver wool and a second layer of cupric oxide, and the second, heated copper.

Preparation of *tertiary* amyl-*t*-alcohol. *Tert.* amyl-*t*-alcohol was prepared from trimethylethylene by means of hydrating with sulphuric acid containing tritium water (6). Three parts by volume of trimethylethylene and one part of 46 % sulphuric acid, containing tritium, were shaken together for two hours, the mixture then becoming homogeneous. It was then neutralized with ammonia and the alcohol phase was washed three times with equal volumes of water in order to remove the tritium in the hydroxyl group. The water was saturated with ammonium sulphate after washing in order to reduce the solubility of the alcohol. The separated alcohol was dried over sodium sulphate and then distilled in a Podbielniak fractionating column, the fraction between 99°–101° taken. The *tert.* amyl alcohol was found to contain less than 0.1 % of the tritium in the alcohol group.

The *trimethylethylene* was prepared from ordinary *tertiary* amyl alcohol (Eastman Kodak, practical, distilled once and the fraction 99°–101° used), which was dehydrated according to a method given by NORRIS and JOUBERT (6).

Preparation of S-*tert.* amyl-*t*-thiuronium picrate. A mixture of 0.5 g of finely powdered thiourea, 1.0 g of *tertiary* amyl bromide and 7.0 ml of *tert.* amyl-*t*-alcohol, to which some mg of water had been added, as a solvent was refluxed for 4.5 hours. 1.5 g of picric acid was then added, and the mixture boiled for 10 minutes, after which the solution was allowed to stand overnight. The crude picrate was filtered, washed with some water and dried. The purification of the product was performed by recrystallisations, twice from 50 % alcohol and then twice from 70 % alcohol, which eventually yielded 0.5 g of the pure picrate as small glistening needles. The melting point is 127°. (Cf. (1) and (2).) (Exp. 1–4.)

The *tertiary* amyl-*t*-alcohol used as the solvent consisted of the *tert.* amyl-*t*-alcohol described above, diluted with twice its volume of ordinary *tert.* amyl alcohol. The small amount of water, 25 mg H_2O per 30.0 ml of diluted alcohol, was added in order to make the conditions exactly similar to those of the control experiments (see below).

The *tertiary amyl bromide* was prepared from *tert.* amyl alcohol and hydrobromic acid in the presence of sulphuric acid.

The experiments 1 and 2 in Table 1 were only of preliminary character.

The *control experiments* were carried out in the same manner as the above experiments with the active alcohol but ordinary *tertiary* amyl alcohol was used as solvent, to which some tritium water had been added (exp. 5–8).

Results and discussion

The results from the preparation of the S-*tert.* amyl thiuronium picrate in *tertiary* amyl-*t*-alcohol as a solvent indicate that there is an approximately complete exchange

Table I. Data of S-*tert.* amyl thiuronium picrates, prepared in *tert.* amyl-*t*-alcohol as solvent.

Exp.	Spec. activity[1]	
	Calc.	Observed
1	14.1	14.2
2	14.1	16.2
3	26.1	24.3
4	26.1	24.8

[1] All figures being average specific activities for the total hydrogen of the molecule.

Table II. Data of S-*tert.* amyl thiuronium picrates, prepared in ordinary *tert.* amyl alcohol, containing tritium water, as solvent.

s_1 = spec. activity averaged over one hydrogen from the amyl group and the hydrogen from the hydroxyl group, thiourea and picric acid.
s_2 = spec. activity averaged over all the hydrogens in the system.

Exp.	Spec. activity		
	Calc.		Observed
	s_1	s_2	
5	34	110	6.6
6	34	110	8.1
7	34	110	7.1
8	34	110	8.1

of radicals. The data are given in Table I, where the experiments 1 and 2 are of preliminary character. The precise determinations in the experiments 3 and 4 show that the activity calculated on the assumption of complete radical exchange is somewhat higher than the found one, and this might be due to a slight hydrogen exchange.

To control that no hydrogen exchange took place during the recrystallisations the last mother liquor from the recrystallisations in experiment 4 was examined for tritium. The activity was too small to be determined accurately, and the hydrogen loss from the amyl group to the bulk of usually exchangeable hydrogens of the system was certainly less than 4.3 % in this recrystallisation.

The results from the control experiments are given in Table II. We see that there is a slight hydrogen exchange, otherwise we would not have recovered any tritium in the S-*tert.* amyl thiuronium picrate. In order to confirm that the activity found was not due to any tritium left at the movable hydrogens of the thiourea group, the final mother liquor from the recrystallisations in experiment 8 was examined for tritium. It was found to contain none.

If we suppose that only one hydrogen from the amyl group besides the hydrogen from the hydroxyl group, thiourea and picric acid took part in the exchange, we get a calculated activity of 34. This value is the most unfavourable one. If all the hydrogen atoms in the reaction mixture took part in the exchange, we should get a calculated activity of 110. The observed value, however, is about 8.

In spite of this hydrogen exchange, we are able to elucidate the reaction mechanism, and find that the tertiary amyl radicals from the halide and the alcohol interchange with each other approximately to equilibrium. The earlier proposed reaction theory is thus strongly supported.

Though these experiments have yielded required information and clearly indicated radical exchange, they would have been more satisfactory, had they been carried out with C^{14} instead of tritium. Our disposal of actual material, however, made it necessary for us to choose tritium as the tracer atom.

The authors are indebted to Professor ARNE FREDGA and Docent LARS MELANDER for many valuable discussions.

SUMMARY

The S-*tert.* amyl thiuronium picrate has been prepared with *tertiary* amyl-*t*-alcohol as a solvent and was found to contain tritium in the tertiary alkyl group. Approximate exchange equilibrium of radicals of the same kind has thus been observed.

Control experiments have been carried out, which indicate that the exchange of radicals is much more rapid than the spontaneous hydrogen exchange, taking part to a slight extent.

The results obtained support the earlier proposed reaction mechanism.

Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala and Nobel Institute of Chemistry, Stockholm 50, November 1953.

REFERENCES

1. L. SCHOTTE, Arkiv Kemi *5* (1952) no. 2.
2. L. SCHOTTE and S. VEIBEL, Acta Chem. Scand. *7* (1953), 1357.
3. F. WILD, Characterisation of Organic Compounds, p. 42 and p. 48, Cambridge (1947).
4. L. MELANDER, Acta Chem. Scand. *2* (1948), 440.
5. —— Arkiv Kemi *2* (1950) no. 12.
6. J. F. NORRIS and J. M. JOUBERT, J. Am. Chem. Soc. *49* (1927), 873.

Tryckt den 1 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 9

Communicated 25 November 1953 by ARNE FREDGA

The acidolysis of ethyl diacetoacetate

II. The effect of cobaltic tris-acetylacetonate

By ARNE BRÄNDSTRÖM

With 3 figures in the text

In a recent paper (1) the present author described the reaction of ethyl diacetoacetate with acetic acid, using various metal derivatives as catalysts, and called attention to the rule that metal derivatives will act as catalysts in this reaction only if the coordination number differs from twice the valence. A further study of this reaction showed that if cobaltic tris-acetylacetonate was added to a mixture of acetic acid and ethyl diacetoacetate at 120°, carbon dioxide evolved, rather slowly at first, but at a gradually increasing rate; so that at first sight this cobalt III compound appeared to be a good catalyst. Since this finding conflicted with the above-mentioned rule, the reaction was studied more closely.

Since the reaction appeared to be autocatalytic, and since cobalt II compounds are good catalysts, it seemed very plausible to postulate a slow reduction of the cobalt III to a cobalt II compound, which was the actual catalyst. As the concentration of the added catalyst was low, this reduction does not measurably affect the concentration of the other reagents.

In order to prove this mechanism the formation of cobalt II compounds had to be demonstrated. Due to the small amounts of the metal compounds present in the reaction mixture, a colorimetric method seemed to be the most appropriate for this purpose. As cobaltic tris-acetylacetonate is very strongly green whereas the cobaltous compound has only a faint reddish tint, the presence of small amounts of cobaltous compounds cannot be estimated directly when cobaltic compounds are present. The concentration of cobaltic compound can, however, be readily estimated. From the decrease in this concentration, that of the cobaltous compound can be calculated.

To test the very plausible assumption that the cobaltic tris-acetylacetonate was broken down into cobaltous compounds, the reaction of ethyl diacetoacetate with acetic acid was studied kinetically with cobaltic tris-acetylacetonate as the catalyst. As the concentration of cobaltous compound present at different times can be calculated from the results obtained with the colorimetric method, and the carbon dioxide formed can be accurately measured, it must be possible to calculate the velocity constant for the reaction of ethyl diacetoacetate with acetic acid, using cobaltous compounds as the catalyst, even if they are added in the form of cobaltic tris-acetylacetonate. If the whole of the catalytic effect is due to cobaltous compounds formed, the velocity constant thus obtained must be the same as that obtained when cobaltous compounds are added in the form of cobaltous acetate. This was found to be the case. Accordingly the catalytic effect of cobaltic tris-acetylacetonate in this reaction is due solely to its tendency to decompose into cobaltous compounds, which are good catalysts.

Experimental

The kinetic tests were performed at 120° in the same way as described previously (1). In the colorimetric measurements 2.00 ml was pipetted out from the hot reaction solution at fixed intervals and diluted with glacial acetic acid to 50 ml. The extinction of this solution was measured in a 4 cm cell at 120 mμ, where the molar extinction of the cobaltic compound was 25 times that of the cobaltous compound.

The formation of cobaltous compounds from cobaltic tris-acetylacetonate

The colorimetric measurements showed that the reaction velocity of the decomposition of the cobaltic compound was proportional to the latter's concentration. The rate of this reaction must then very probably follow a first order kinetic. However, measurement was made more difficult by discoloration of the reaction mixture during the reaction. Fortunately this discoloration proved to be slight and reproducible from one experiment to another, regardless of the amount of cobaltic tris-acetylacetonate added. This fact makes it possible to correct the values obtained for this factor in the following way.

For the rate of change in the measured extinction, E, the following equation is valid:

$$\text{I} \qquad \frac{dE}{dt} = -C_{\text{Co}^{3+}} \cdot K' + K''$$

where K' and K'' are velocity constants and $C_{\text{Co}^{3+}}$ the concentration of cobaltic compounds present.

Table 1

$C_0 = 0.289$ m. mol.		$C_0 = 0.547$ m. mol.		$C_0 = 0.796$ m. mol.	
t	E	t	E	t	E
60	0.285	60	0.540	60	0.766
120	0.276	120	0.519	120	0.753
180	0.263	180	0.484	180	0.702
240	0.248	240	0.444	240	0.669
300	0.235	300	0.424	300	0.624
360	0.226	360	0.394	360	0.576
420	0.215	420	0.374	420	0.533
$K^* = 2.06 \cdot 10^{-4}$		$K^* = 4.72 \cdot 10^{-4}$		$K^* = 7.24 \cdot 10^{-4}$	

Table 2

C_0	0.289	0.547	0.796
$1/C_0$	3.46	1.83	1.26
K^*/C_0	$7.13 \cdot 10^{-4}$	$8.64 \cdot 10^{-4}$	$9.12 \cdot 10^{-4}$
$K'' = 9.3 \cdot 10^{-5}$			

If $C_{Co^{3+}}$ as a first approximation is considered to be constant $= C_0$, equation I can easily be integrated, yielding:

$$\text{II} \qquad E - E_0 = (K'' - C_0 K')t \qquad E_0 = E \text{ at the time } t = 0.$$

A plot of E against t must then give a straight line, the slope being $-K^* = K'' - C_0 K'$. Taking different C_0 values, it will be seen that if $-K^*/C_0$ is plotted against $1/C_0$ a straight line will result, the slope of which is K''. The results thus obtained are given in Tables 1 and 2.

From the values obtained for K'' the extinction value for the cobaltic compound $E_{Co^{3+}}$ can be calculated at different times by the formula III based on the fact that the molar extinction of cobaltous compounds is 1/25 of that of cobaltic.

$$\text{III} \qquad E_{Co^{3+}} = E - \frac{25}{24} K'' \cdot t - \frac{E_0 - E}{24} = E - E_{\text{corr.}}$$

In this formula E_0 is taken as the intercepts of the lines obtained by plotting E against t, as reproducible values could not be obtained at the beginning of the reaction.

From the values for $E_{Co^{3+}}$ thus obtained, we can calculate the velocity constant K_1 for the reaction $Co^{3+} \rightarrow Co^{2+}$ from the formula

$$\text{IV} \qquad \frac{d E_{Co^{3+}}}{dt} = -K_1 \cdot E_{Co^{3+}}$$

Table 3

	$C_0 = 0.289$ m. mol.			$C_0 = 0.547$ m. mol.			$C_0 = 0.796$ m. mol.		
min.	E	$E_{Co^{3+}}$	log $E_{Co^{3+}}$	E	$E_{Co^{3+}}$	log $E_{Co^{3+}}$	E	$E_{Co^{3+}}$	log $E_{Co^{3+}}$
0 . . .	0.300	0.300	$\bar{1}.477$	0.572	0.572	$\bar{1}.757$	0.836	0.836	$\bar{1}.922$
60 . . .	0.285	0.279	$\bar{1}.466$	0.540	0.533	$\bar{1}.727$	0.766	0.757	$\bar{1}.879$
120 . . .	0.276	0.263	$\bar{1}.420$	0.519	0.505	$\bar{1}.703$	0.753	0.738	$\bar{1}.868$
180 . . .	0.263	0.244	$\bar{1}.387$	0.484	0.463	$\bar{1}.666$	0.702	0.679	$\bar{1}.832$
240 . . .	0.248	0.223	$\bar{1}.348$	0.444	0.415	$\bar{1}.618$	0.669	0.639	$\bar{1}.806$
300 . . .	0.235	0.203	$\bar{1}.308$	0.424	0.389	$\bar{1}.590$	0.624	0.586	$\bar{1}.768$
360 . . .	0.226	0.188	$\bar{1}.274$	0.394	0.352	$\bar{1}.547$	0.576	0.530	$\bar{1}.724$
420 . . .	0.215	0.171	$\bar{1}.233$	0.374	0.325	$\bar{1}.512$	0.533	0.480	$\bar{1}.681$
	$K_1 = 1.45 \cdot 10^{-3}$			$K_1 = 1.44 \cdot 10^{-3}$			$K_1 = 1.42 \cdot 10^{-3}$		

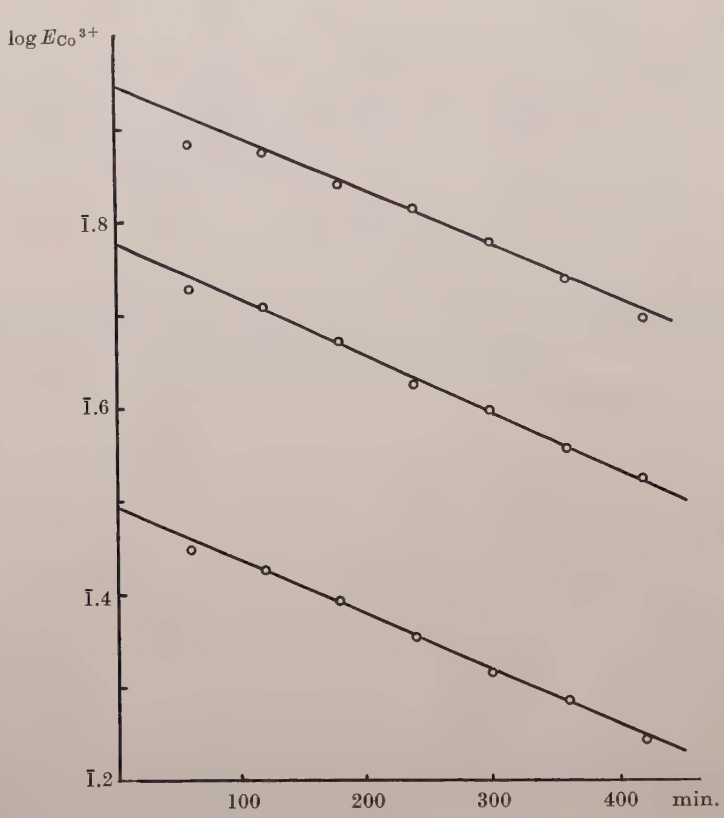


Fig. 1.

from which K_1 is readily calculated. The results obtained are shown in Table 3 and Fig. 1.

If cobaltous acetate is added as the catalyst, the following differential equation will be valid:

$$\text{V} \qquad \frac{dx}{dt} = K_2 (a-x)(b-x)\sqrt{C_{Co^{2+}}},$$

a and b being the initial molar fractions of glacial acetic acid and ethyl diacetoacetate respectively, and x the molar fraction corresponding to the carbon dioxide evolved.

From this equation K_2 is readily calculated. The results obtained are given in Tables 4–6 and Fig. 2.

If cobaltic tris-acetylacetonate is added, the same differential equation V must be valid if the cobaltous compounds formed are the only catalysts present. This equation can be integrated if the concentration of cobaltous compounds $C_{Co^{2+}}$ can be obtained at different times. This is possible by means of equation IV, which can be integrated and rearranged to

$$\text{VI} \qquad C_{Co^{2+}} = C_0 (1 - e^{-K_1 t}).$$

Table 4

min.	ml CO_2	x	$a-x$	$b-x$	$\log\frac{a-x}{b-x}$
0	0	—	0.6667	0.3333	0.3010
20	33	0.0045	0.6622	0.3288	0.3041
40	74	0.0101	0.6566	0.3232	0.3078
60	116	0.0159	0.6508	0.3174	0.3119
80	157	0.0215	0.6452	0.3118	0.3158
100	197	0.0269	0.6398	0.3064	0.3197
120	236	0.0323	0.6344	0.3010	0.3238
140	274	0.0374	0.6293	0.2959	0.3278
160	312	0.0426	0.6241	0.2907	0.3318
180	352	0.0481	0.6186	0.2852	0.3362
200	385	0.0526	0.6141	0.2807	0.3400
220	420	0.0574	0.6093	0.2759	0.3440
240	451	0.0616	0.6051	0.2717	0.3477
260	486	0.0664	0.6003	0.2669	0.3520
280	518	0.0708	0.5959	0.2625	0.3561
300	548	0.0749	0.5918	0.2584	0.3599
320	578	0.0790	0.5877	0.2543	0.3638
340	612	0.0836	0.5831	0.2497	0.3683
360	642	0.0877	0.5790	0.2456	0.3725
380	672	0.0918	0.5749	0.2415	0.3767

29.00 mg $Co(OCOCH_3)_2 = 0.1638$ m. mol. $V_\infty = 2439$ ml. $K_2 = 3.43 \cdot 10^{-3}$

Table 5

min.	ml CO_2	x	$a-x$	$b-x$	$\log\frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
20	41	0.0056	0.6611	0.3277	0.3048
40	94	0.0129	0.6538	0.3204	0.3097
60	150	0.0206	0.6461	0.3127	0.3152
80	202	0.0277	0.6390	0.3056	0.3203
100	256	0.0351	0.6316	0.2982	0.3259
120	310	0.0424	0.6243	0.2909	0.3317
140	358	0.0489	0.6178	0.2844	0.3370
160	407	0.0556	0.6111	0.2777	0.3425
180	452	0.0618	0.6049	0.2715	0.3479
200	498	0.0681	0.5986	0.2652	0.3536
220	542	0.0741	0.5926	0.2592	0.3592
240	582	0.0795	0.5872	0.2538	0.3643
260	624	0.0853	0.5814	0.2480	0.3700
280	662	0.0905	0.5762	0.2428	0.3753
300	700	0.0957	0.5710	0.2376	0.3807
320	737	0.1007	0.5660	0.2326	0.3862

50.80 mg $Co(OCOCH_3)_2 = 0.2870$ m. mol. $V_\infty = 2431$ ml. $K_2 = 3.53 \cdot 10^{-3}$

Table 6

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
20	64	0.0086	0.6581	0.3247	0.3068
40	139	0.0186	0.6481	0.3147	0.3137
60	214	0.0287	0.6380	0.3046	0.3211
80	283	0.0379	0.6288	0.2954	0.3281
100	353	0.0473	0.6194	0.2860	0.3356
120	419	0.0562	0.6105	0.2771	0.3431
140	485	0.0650	0.6017	0.2683	0.3508
160	544	0.0729	0.5938	0.2604	0.3580
180	602	0.0807	0.5860	0.2526	0.3655
200	658	0.0882	0.5785	0.2451	0.3730
220	711	0.0953	0.5714	0.2380	0.3803
240	761	0.1020	0.5647	0.2313	0.3876
260	810	0.1086	0.5581	0.2247	0.3951
280	855	0.1146	0.5521	0.2187	0.4021
300	901	0.1208	0.5459	0.2125	0.4097
320	943	0.1264	0.5403	0.2069	0.4168
340	982	0.1317	0.5350	0.2016	0.4239

91.93 mg $Co(OCOCH_3)_2 = 0.5193$ m. mol. $V_\infty = 2486$ ml. $K_2 = 3.53 \cdot 10^{-3}$

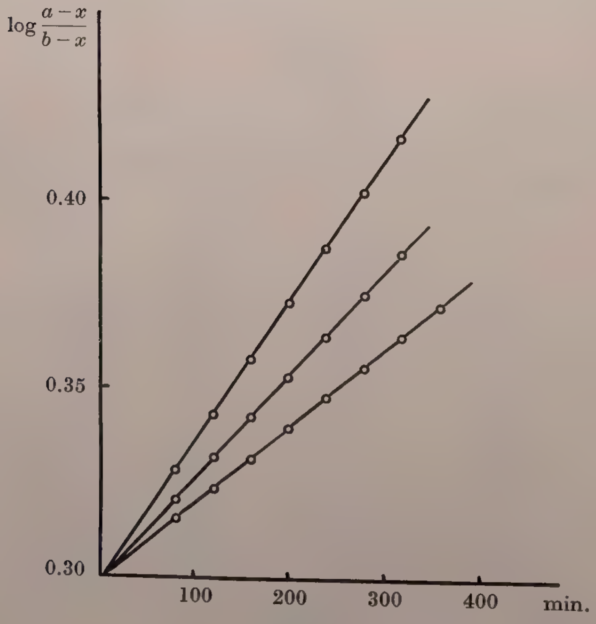


Fig. 2.

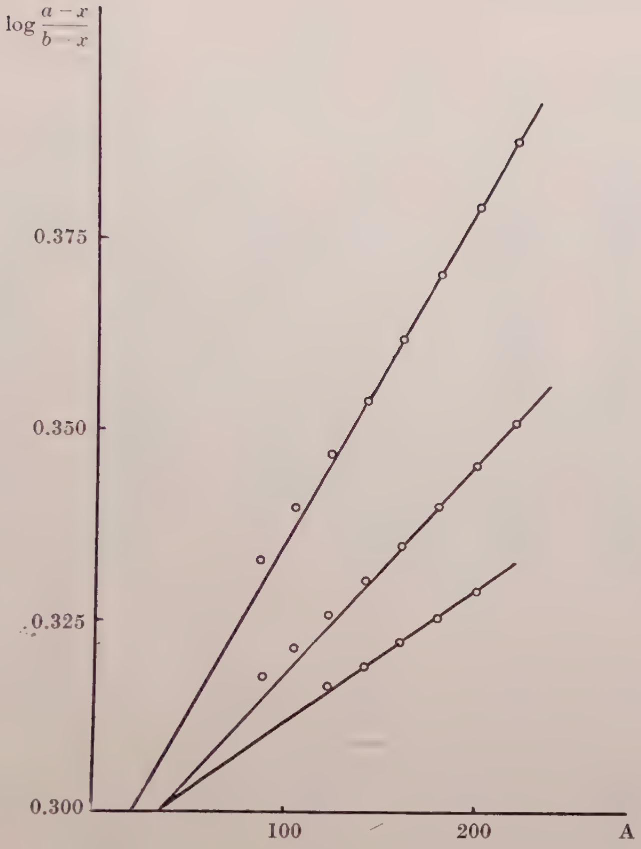


Fig. 3.

By incorporating this value into equation V and rearranging, we obtain:

VII
$$\frac{dx}{(a-x)(b-x)} = K_2 \sqrt{C_0 (1 - e^{-K_1 t})}\, dt$$

or, on integration:

$$\frac{1}{a-b} \ln \frac{b(a-x)}{a(b-x)} = K_2 \sqrt{C_0} \frac{1}{K_1} \left[\ln \frac{1+\sqrt{1-e^{-K_1 t}}}{1-\sqrt{1-e^{-K_1 t}}} - 2\sqrt{1-e^{-K_1 t}} \right].$$

A plot of $\log a-x/b-x$ against $A = 1/K_1 \left[\ln \frac{1+\sqrt{1-e^{-K_1 t}}}{1-\sqrt{1-e^{-K_1 t}}} - 2\sqrt{1-e^{-K_1 t}} \right]$ should thus give a straight line, the slope being $\frac{K_2 \sqrt{C_0}(a-b)}{2.303}$. From this, K_2 is readily obtainable.

Table 7

min.	A	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0	0		0.6667	0.3333	0.3010
30	4.1	5	0.0007	0.6660	0.3326	0.3014
60	11.5	12	0.0017	0.6650	0.3316	0.3022
90	21.3	23	0.0032	0.6635	0.3301	0.3031
120	32.5	36	0.0049	0.6618	0.3284	0.3043
150	45.0	54	0.0074	0.6593	0.3259	0.3060
180	58.8	73	0.0100	0.6567	0.3233	0.3078
210	73.7	90	0.0123	0.6543	0.3210	0.3093
240	89.4	113	0.0155	0.6512	0.3178	0.3115
270	105.9	138	0.0189	0.6478	0.3144	0.3139
300	123.2	165	0.0226	0.6441	0.3107	0.3167
330	141.6	193	0.0264	0.6403	0.3069	0.3194
360	160.4	223	0.0306	0.6361	0.3027	0.3225
390	179.6	256	0.0350	0.6317	0.2983	0.3258
420	199.6	288	0.0395	0.6272	0.2938	0.3293

45.5 mg $Co(CH_3COCHCOCH_3)_3 = 0.1277$ m. mol. $V_\infty = 2433$ ml. $K_2 = 3.39 \cdot 10^{-3}$

Table 8

min.	A	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0	0	0	0.6667	0.3333	0.3010
30	4.1	6	0.0008	0.6659	0.3325	0.3016
60	11.5	20	0.0027	0.6640	0.3306	0.3029
90	21.3	37	0.0049	0.6618	0.3284	0.3043
120	32.5	54	0.0072	0.6595	0.3261	0.3058
150	45.0	82	0.0109	0.6558	0.3224	0.3084
180	58.8	114	0.0152	0.6515	0.3181	0.3112
210	73.7	146	0.0195	0.6472	0.3138	0.3143
240	89.4	184	0.0246	0.6421	0.3087	0.3181
270	105.9	223	0.0297	0.6370	0.3036	0.3218
300	123.2	263	0.0351	0.6316	0.2982	0.3259
330	141.6	308	0.0411	0.6256	0.2922	0.3306
360	160.4	352	0.0469	0.6198	0.2864	0.3353
390	179.6	398	0.0532	0.6135	0.2801	0.3405
420	199.6	445	0.0594	0.6073	0.2739	0.3458
450	220.2	493	0.0657	0.6010	0.2676	0.3514

108.1 mg $Co(CH_3COCHCOCH_3)_3 = 0.3034$ m. mol. $V_\infty = 2498$ ml. $K_2 = 3.42 \cdot 10^{-3}$

Table 9

min.	A	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0	0		0.6667	0.3333	0.3010
30	4.1	22	0.0029	0.6638	0.3304	0.3030
60	11.5	48	0.0064	0.6603	0.3269	0.3053
90	21.3	84	0.0112	0.6555	0.3221	0.3086
120	32.5	124	0.0165	0.6502	0.3168	0.3123
150	45.0	173	0.0230	0.6437	0.3103	0.3169
180	58.8	222	0.0296	0.6371	0.3037	0.3218
210	73.7	275	0.0366	0.6301	0.2967	0.3271
240	89.4	332	0.0442	0.6225	0.2891	0.3330
270	105.9	394	0.0524	0.6143	0.2809	0.3399
300	123.2	456	0.0608	0.6059	0.2725	0.3470
330	141.6	516	0.0687	0.5980	0.2646	0.3541
360	160.4	584	0.0777	0.5890	0.2556	0.3625
390	179.6	648	0.0863	0.5804	0.2470	0.3710
420	199.6	712	0.0949	0.5718	0.2384	0.3799
450	220.2	773	0.1029	0.5638	0.2304	0.3886

292.9 mg $Co(CH_3COCHCOCH_3)_3 = 0.8222$ m. mol. $V_\infty = 2504$ ml. $K_2 = 3.39 \cdot 10^{-3}$

The results obtained are shown in Tables 7–9 and Fig. 3. Due to repression of the rate of formation of cobaltous compounds at the beginning of the reaction, the best values for K_2 are obtained from the slopes of the asymptotes to the curves in Fig. 3. These values are given in Tables 7–9.

The author is indebted to Messrs. Pharmacia Ltd. for permission to publish this paper and to Fil. Mag. Solveig Hansen for assistance with the experimental work.

Research Laboratory of Pharmacia Ltd., Uppsala, November 1953.

REFERENCE

1. A. Brändström, Arkiv f. Kemi 6, 201 (1953).

Tryckt den 4 mars 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 10

Communicated 25 November 1953 by H. v. EULER and K. MYRBÄCK

On the isolation of two types of intermediates from the Strecker degradation

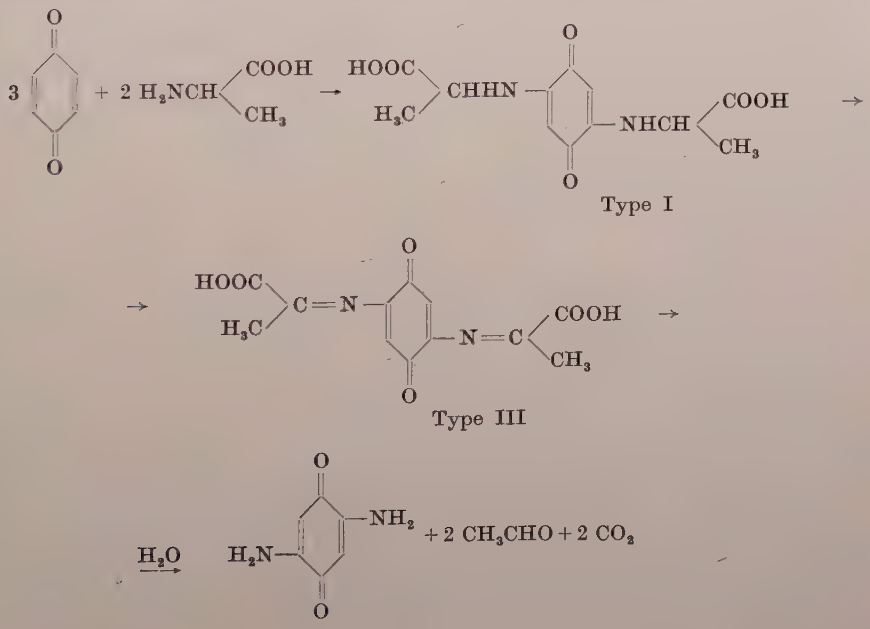
By STIG ÅKERFELDT

With 2 figures in the text

STRECKER (1) found in 1862 that alanine reacts with alloxane forming an aldehyde and CO_2. It was later found by SCHÖNBERG et al. (2) that many carbonyl compounds react with amino acids in a similar manner. Therefore these authors proposed the name "The STRECKER Degradation" for this reaction.

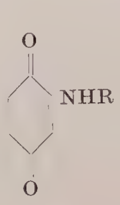
The following work was carried out in order to investigate the possibilities of a reaction between two hypothetical intermediates from the degradation of N,N-dimethyl-4-aminoazobenzene in rats—namely 1,4-benzoquinone and 1,4-benzoquinonimine—and α-amino acids, dipeptides and proteins.

WIELAND and BERGEL (3) have studied the reaction between alanine and 1,4-benzoquinone in water. They obtained acetaldehyde and CO_2 and proposed the following scheme for the degradation of the α-amino acid:



No intermediates, however, were isolated but the authors referred to FISCHER and SCHRADER (4) who isolated esters of compounds similar to type I from reactions between α-amino acid esters and 1,4-benzoquinone.

From 4-aminobenzoic acid and 1,4-benzoquinone SUCHANEK (5) obtained a substance similar to type I but also a substance of a mono-type, II, with only one amino acid molecule attached to the quinone ring:



Type II

1,4-benzoquinone and α-amino acids. Throughout this investigation the reactions between α-amino acids and 1,4-benzoquinone were carried out in boiling 50 % ethanol-water. If the time of reaction is kept short enough (max. 5 minutes) intermediates of the STRECKER degradation can be isolated.

The WIELAND-BERGEL scheme (type I) requires 3 moles of quinone and 2 moles of the amino acid. The compounds of the mono type II require the mole proportions 2:1. These two possibilities have been investigated by the writer and in all cases the substances obtained consisted of a mixture of the mono- and di-types with a marked dominance for the mono-type II. The yields were better when the molar proportions were 2:1. Fig. 1 shows the rate of formation of 2-glycino-benzoquinone-1,4 in water at 20°C.

The isolated compounds of type II are very sparingly soluble in the usual solvents and they are therefore very difficult to recrystallize. They are soluble in NaOH and can be partly purified by repeated precipitating from their alcaline solution by mineral acids.

The lead salts of II are very sparingly soluble in water and can be precipitated from a methanolic solution of II by lead acetate.

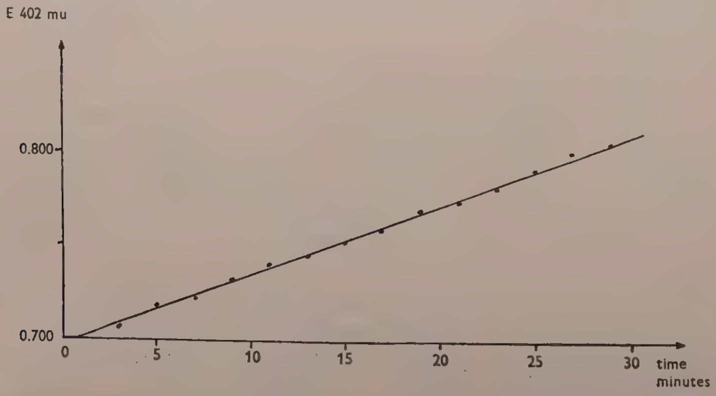


Fig. 1. Rate of formation of 2-glycino-benzoquinone-1,4 in water at 20°C 40 mC of 1,4-benzoquinone and 20 mC of glycine.

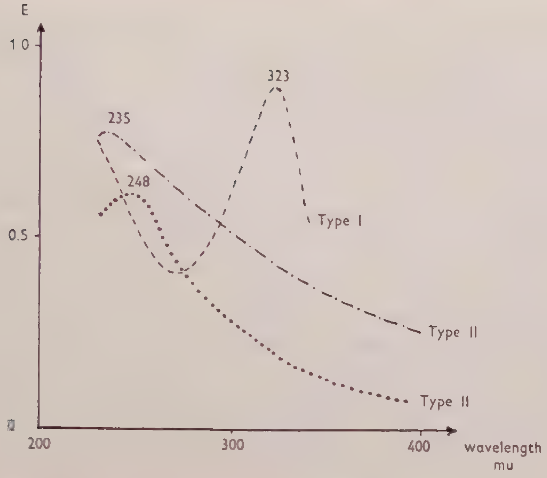


Fig. 2. Absorption spectra of: – · – · – · = 2-(DL-α-alanino)-benzoquinone-1,4 in 0.2n NaOH. – – – – – = 2,5-diglycylglycino-benzoquinone-1,4 in 0,2n NaOH. . . . . . . = β-phenylalanino-benzoquinonimine-1,4 in 0.2n NaOH.

The substances of the mono-type are characterized by their deep red or black colour and their high melting points. They form dioximes and form derivatives with 2,4-dinitrochlorobenzene; they are stabile in cold solutions of H_3O^+ and OH^-. The substances are easily reduced to the corresponding hydroquinone derivatives, which, however, are very instable and easily reoxidized even by air, especially in alkaline solution.

In aqueous solution II slowly decomposes and CO_2 is evolved. No aldehyde is formed, however, unless 1,4-benzoquinone is present. This fact and the one that II formes a derivative with 2,4-dinitrochlorobenzene excludes these compounds from beeing of type III (p. 75).

UV spectra of these compounds in alcaline solution show absorption maxima between 235–250 mμ (Fig. 2.)

1,4-benzoquinone and glycylglycine, casein. One dipeptide, glycylglycine, was investigated and proved to undergo the STRECKER degradation. The compound isolated was of type 1 and shows quite a different UV spectrum from the compounds of type II. (Fig. 2.)

Except of the spectrum the diglycylglycinoquinone formed shows the same characteristics and reactions as the mono amino acid quinones.

Spectra of the crude mono-types II show a very weak absorption around 320 mμ, which probably is due to the formation of small quantities of the di-types I as these seem to give absorption around this wavelength (Fig. 2).

The reaction between casein and 1,4-benzoquinone was investigated at room temperature. No CO_2 was evolved. The spectrum of the compound formed only showed the absorption maxima of casein and 1,4-benzoquinone. Therefore it is very likely that casein sorbes the quinone without the formation of compounds similar to the types I or II.

This finding is in agreement with the X-ray investigation of casein fibres treated with 1,4-benzoquinone made by DUCZKO (6) and the investigation by WALDSCHMIDT-LEITZ (7).

1,4-benzoquinonimine and α-amino acids. The main difficulties in carrying out reactions between 1,4-benzoquinonimine and α-amino acids depend on the facts that the imine is unstable in the presence of water and that the α-amino acids are very sparingly soluble in organic solvents. Successful reaction could only be carried out in one case, namely with β-phenylalanine. The solvent used consisted of benzene, abs. ethanol and water in the proportions 5:8:2 (v:v:v). This low water content did not affect the imine appreciably. The substance isolated proved to be of type II. The UV spectrum of it is similar to those of the quinone derivatives of type II (Fig. 2). A very weak peak at 360 mμ in the spectrum of the crude product is probably due to a compound of the di-type I.

1,4-benzoquinonimine has mainly been prepared according to a method by WILLSTÄTTER and PFANNENSTIEL (8). Instead of ether as a solvent, however, the writer used dry benzene. This solvent proved to be a better one than ether as the time of reaction could be cut down to 30 minutes instead of 2 hours and the yield was still found to be approximately 100 %.

Experimental

2-glycino-benzoquinone-1,4. 2.00 g of glycine and 4.32 g of 1,4-benzoquinone (mole prop. 2:3) were refluxed for 5 minutes in 50 ml of ethanol-water, v/v. After cooling the precipitate was filtered off and dissolved in 2n NaOH and reprecipitated with 4n H_2SO_4. This purification process was repeated 4 times. Finally the precipitate was washed 3 times with water. The purification process was carried out in centrifuge tubes and instead of filtering the centrifuge was used to avoid loss of material during the process. The dry substance melts >300°C. Yield: ca. 20 %. When the mole proportions 1:2 are used the yield is about 30 %. Abs. max. in 0.2n NaOH: 240 mμ.

$C_8H_7NO_4$ (181.1): calc. N: 7.74 %; found N: 7.68 %.

The lead salt was prepared by dissolving the base-acid purified substance in hot methanol to which an excess of aqueous lead acetate was added. The precipitate was centrifuged off and washed several times with hot methanol and distilled water.

$C_{16}H_{12}N_2O_8Pb$ (567.3): calc. C: 33.8 %, H: 2.13 %, Pb: 36.5 %; found C: 34.01 %, H: 2.23 %, Pb: 36.8 %.

2-(DL-α-alanino)-benzoquinone-1,4. 1.50 g of DL-α-alanine and 2.73 g of 1,4-benzoquinone were refluxed for 5 minutes in 50 ml of ethanol-water, v/v. After cooling the black precipitate was centrifuged off and purified as described above. Mp >300°C. Yield: 12 % (mole prop. 1:2 yield ca 15 %). Abs. max. in 0.2n NaOH: 235 mμ.

Lead salt. $C_{18}H_{16}N_2O_8Pb$ (595.5): calc. C: 35.3 %, H: 2.71 %, Pb: 34.8 %; found C: 35.19 %, H: 2,88 %, Pb: 34.5 %.

2-(L-leucino)-benzoquinone-1,4. 2.00 g of L-leucine and 2.48 g of 1,4-benzoquinone (mole prop. 2:3) were refluxed for 5 minutes in 50 ml of ethanol-water, v/v. After cooling the deep violet precipitate was centrifuged off and purified as described above. Mp >300°C. Yield: 5 % (mole prop. 1:2 yield ca. 20 %). Abs. max. in 0.2n NaOH: 250 mμ.

Lead salt. $C_{24}H_{28}N_2O_8Pb$ (679.7): calc. C: 42.4 %, H: 4.15 %, Pb: 30.5 %; found C: 41.68 %, H: 3.70 %, Pb: 30.9 %.

2,5-diglycylglycino-benzoquinone-1,4. 2.00 g of glycylglycine and 2.46 g of 1,4-benzoquinone (mole prop. 2:3) were refluxed for 5 minutes in 50 ml of ethanol-water, v/v. After cooling the black precipitate was centrifuged off and purified as described above. The substance begins to decompose at 253–6°C. Mp > > 300°C. Yield: 20 %. Abs. max. in 0.2 n NaOH: 323 mμ. Equiv. weight: calc. 184; found 180 ± 5.

$C_{14}H_{16}O_8N_4$ (368.3): calc. C: 45.7 %, H: 4.38 %; found C: 45.92 %, H: 4,81 %.

Lead salt. $C_{14}H_{14}O_8N_4Pb$ (573.5): calc. C: 29.4 %, H: 2.44 %, Pb: 36.2 %; found C: 30.02 %, H: 2.86 %, Pb: 36.5 %.

Casein and 1,4-benzoquinone. 1.00 g of casein was dissolved in 80 ml of 0.2n NaOH and 0.50 g of 1,4-benzoquinone were added. The solution instantly turned brown, which indicates an instant reaction or sorption as the solution of 1,4-benzoquinone in OH^- is green in colour. After 10 minutes of intense shaking at 20°C the reaction mixture was acidified with 4n H_2SO_4 and centrifuged. During the processes no CO_2 was evolved which indicates that no STRECKER degradation takes place.

The brown substance is less soluble in H_3O^+ than is casein. It was partly purified by dissolving in 0.5n NaOH and reprecipitating with 4n H_2SO_4 4 times. Finally the precipitate was washed twice with water.

About 20 mg of the partly purified substance were dissolved in 5 ml of 1n NaOH and were further purified by adsorption column chromatography. SiO_2 (Hyflo Super Cel; diam: 1.2 cm., length: 20 cm.) was used in the column which was developed with 0.05 C H_2SO_4. Speed of flow: 18 drops/min.

Except for impurities that were stopped at the top of the column two bands were obtained. The upper band was yellow and consisted of 1,4-benzoquinone. The lower band was brown. This one was cut out and eluated with 0.1n NaOH and its UV spectrum was determined.

1,4-benzoquinonimine. 1.0 g of 4-aminophenol and 2.5 g of silver oxide (mole prop. 1: 1,2) were mixed in 200 ml of dry benzene containing 5 g of waterfree sodium sulfate. The mixture was shaken in N_2-atmosphere for 30 minutes and filtered. A light yellow benzene solution was obtained which was stable for 24 hours at 4°C in N_2-atmosphere. The remaining silver fraction did not contain unreacted 4-aminophenol.

1,4-benzoquinonimine and β-phenylalanine. To 150 ml of dry benzene containing 1.0 g of 1,4-benzoquinonimine were added 240 ml of abs. ethanol, 60 ml of water and 1.6 g of β-phenylalanine (mole prop. 1:1). The mixture was refluxed for 15 minutes and evaporated to dryness at 20°C. The black crystalline mass obtained contained to about 50 % unreacted β-phenylalanine. The mass was shaken with hot abs. ethanol, wherein only the reaction product is soluble. The substance was purified by the base-acid method described above. Yield: 0.5 g (20 %). The substance begins to decompose around 120°C and melts at 140–145°C. It forms an oxime which decomposes at 242–245°C. Abs. max. in 0.2n NaOH: 248 mμ.

Lead salt of β-phenylalanino-benzoquinonimine-1,4. The lead salt was prepared as described above.

$C_{30}H_{26}N_4O_6Pb$ (745.8): calc. C: 48.4 %, H: 3.49 %, Pb: 27.8 %; found C: 48,77 %, H: 3.69 %, Pb: 28.0 %.

Discussion

An extensive work has been done to investigate the possibilities of further purification of the compounds of types I and II. As mentioned above, however, the substances are very difficult to recrystallize according to their very low solubility. Consequently adsorption column chromatography was not found applicable depending on the large amounts of solvent that must be used in applying the substances on the column.

Other metal salts of the compounds were investigated but no ones were found easier to purify than the Pb-salts.

Different derivatives of types I and II—as the addition compounds with bromine—were also investigated, but these were found to be less stable than the original compounds and consequently not suitable for purification.

Esterification (the FISCHER-SPEIER method) gives extremely low yields. However, the figures of analysis obtained are accurate enough for deciding wether the compounds formed are of type I or II.

Summary

1. From the reactions between three α-amino acids and 1,4-benzoquinone (the STRECKER Degradation) intermediates have been isolated. They consist of compounds with one α-amino acid molecule N-bound to the quinone ring (type II).

2. From the reaction between glycylglycine and 1,4-benzoquinone (which is of the STRECKER degradation type) an intermediate was isolated. It consists of two glycylglycine molecules N-bound to the quinone ring (type I).

3. Casein does not undergo the STRECKER degradation in alcaline solution with 1,4-benzoquinone.

4. β-Phenylalanine proved to undergo the STRECKER degradation in the presence of 1,4-benzoquinonimine. An intermediate of type II was isolated.

University of Stockholm, Institute for Research in Organic Chemistry.

REFERENCES

(1) STRECKER, W., Ann. *123* (1862), 363.
(2) SCHÖNBERG, A., MOUBASHER, R., MOSTAFA, A., J. Chem. Soc. *1948*, 176–82.
(3) WIELAND, H., BERGEL, F., Ann. *439* (1925), 206.
(4) FISCHER, E., SCHRADER, H., Ber. 43 (1910), 525.
(5) SUCHANEK, O. J., Prakt. Chem. *90* (1914), 467.
(6) DUCZKO, K., Przemysl Chem. *21* (1937), 258–61.
(7) WALDSCHMIDT-LEITZ, E., Die Chemie *55* (1942), 62–3.
(8) WILLSTÄTTER, R., PFANNENSTIEL, A., Ber. *37* (1904), 4607.

Tryckt den 4 mars 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 11

Communicated 27 January 1954 by ARNE FREDGA and GUNNAR HÄGG

Chelate alkali compounds in organic reactions

II. A kinetic study of the alkylation of different alkali compounds of ethyl acetoacetate

By ARNE BRÄNDSTRÖM

With 1 figure in the text

In the first paper in this series (1) the writer propounded a theory of the alkylation of metallic derivatives of tautomeric substances. The present paper is concerned with a kinetic investigation on the effect of a variation of the alkali cation in the alkylation of ethyl acetoacetate. This investigation has clearly demonstrated the existence of a chelate alkylation mechanism together with an ionic one.

In the alkylation of e.g. sodium acetylacetonate (with a chelation constant $K_c = 10^{3.2}$ in water-dioxane (2)) a purely ionic reaction seems almost as unlikely as a purely chelate reaction of the tetramethylammonium compound of diethyl malonate. It is plausible, therefore, to consider the alkylation as a result of two simultaneous reactions, one being ionic, the other chelate. In order to see in which reactions the ionic or the chelate mechanism is dominant, these two reactions have to be defined. For this purpose the ionic reaction is defined as a reaction entirely independent of the metal ion present in the solution, and the chelate mechanism as a reaction within a complex containing both the mesomeric anion and the alkyl halide coordinated to the cation present.

This definition may appear to be arbitrary, but it is convenient and both extremes are clearly defined reactions which may be obtained under suitable conditions. It does not necessarily mean that there are only two distinct mechanisms operating, but rather a continuous series of mechanisms varying between these two extremes; in the same way as the position of the cation may vary from that in the chelate to one in which it is totally separated from the anion. This series of reactions can, however, be expressed in terms of the two extremes; just as the properties of the solution can be expressed in terms of the chelate and the ionic compound.

The theoretical treatment of the ionic reaction is very simple. For a given reaction it has a velocity constant K_2 regardless of the cation present.

The chelate mechanism is much more complicated, since the cation has an influence on both the anion and the alkyl halide present. The effect of the cation can be expected to consist in withdrawal of electrons from the anion and the halide. Since the withdrawal from the anion is probably independent of the properties of the halide, and that from the halide independent of the anion, the two effects can be treated separately. For the mathematical treatment the velocity coefficient K_1' for the first order decomposition of the complex is split into two factors $K_1' = K'\, K''$, where K' refers to a hypothetical reaction in which the cation affects only the anion, and K'' to one in which the cation affects only the halide.

If a variation of the cation does not appreciably affect the resonance in the chelate ring, the effect on K' is only dependent on the ionic radius of the alkali cation, K' being increasing from lithium to cesium. This effect can be quantitatively expressed so that a plot of log K' against $1/r$, where r is the ionic radius, will give a straight line (3).

An electron withdrawing from the halogen atom of the halide will increase the polarisation of that reagent and thus increase the velocity of the reaction. This effect is greater for lithium than for cesium and can be quantitatively expressed so that a plot of log K'' against $1/r$ will give a straight line.

The result of the two factors operating in the decomposition of the complex in the chelate reaction is that a plot of log K_1' against $1/r$ will give a straight line.

The velocity of the chelate reaction is dependent not only on the velocity constant K_1' for the decomposition of the complex containing both the anion and the halide coordinated to the cation, but also on the actual concentration of that complex. This concentration is in turn a function of that of the alkali chelate and the alkyl halide.

If the fraction of the alkali compound present as the chelate is X, that present as the anion $1-X$, and the concentration of the cation m, the equation $X = (1-X)\, m\, K_c$, where K_c is the chelation constant, gives the relative amounts of the anion and the chelate present in the reaction. From this we can see that an increase in both K_c and m favours the chelate reaction. Since the solvation of the cation competes with the chelation, it is obvious that the chelation constant K_c increases with a decreasing coordination tendency of the solvent.

The coordination number of the alkali cation in solvents of high polarity is higher than 2, so that both alkyl halide and solvent molecules can be coordinated to it in the chelate. Since the halide and the solvent compete in this coordination, the concentration of the halide-containing complex increases with a decrease in the coordination tendency of the solvent. The result is that both the concentration of the chelate and its tendency to form the complex compound, assumed in the chelate reaction mechanism, are increased with a decrease in the coordination tendency of the solvent. The choice of the solvent will thus permit wide variation of the relative importance of the chelate and the ionic reaction.

A variation of the cation used gives us a further possibility of varying the relative importance of the chelate and the ionic reaction. This effect can be predicted as follows: If a is the concentration of the halide, b that of the alkali compound ($b\,X$ is the concentration of the chelate), Y the fraction of the chelate present as the halide-containing complex, and K''' a constant, the following equation will be valid:

$$\text{(I)} \qquad \frac{Y}{1-Y} = K'''\,(a - b\,X\,Y).$$

In a dilute alcoholic solution Y is probably small, as the concentration of the halide is small in comparison with that of the solvent, which also has a higher coordination tendency than the halide. Hence, the equation

$$\text{(II)} \qquad Y = K'''\, a$$

gives a close approximation of the equilibrium in a dilute alcoholic solution. Since K''' is the equilibrium constant for the competing electrostatic attractions of the uncharged solvent and halide molecules to the cation. K''' may be expected to be almost constant, even if the ionic radius of the cation is varied, and the small variation in K''' can be expressed so that a plot of log K''' against $1/r$ will give a straight line (3).

According to the above considerations the velocity of the chelate alkylation reaction is $K_1 X a b = K' K'' K''' X a b$, where the velocity constants $K_1 = K' K'' K'''$ obtained for different alkali cations so vary that a plot of log K_1 against $1/r$ will give a straight line. This is, therefore, the characteristic property of a reaction where X is unity; i.e., for a mainly chelate reaction. For an ionic reaction, on the other hand, the velocity constant K_2 is the same for all alkali cations.

To facilitate an understanding of reactions where the alkylation is neither a pure chelate nor a pure ionic reaction, but one in which both these types proceed at the same time, the following mathematical treatment is helpful:

For the alkylation reaction the following equation is valid,

$$\text{(III)} \qquad K a b = K_1 X a b + K_2 (1 - X) a b,$$

where K is the apparent velocity constant. From this we can obtain

$$\text{(IV a)} \qquad K = K_1 - (K_1 - K_2)(1 - X),$$

or rearranged

$$\text{(IV b)} \qquad K = K_2 + (K_1 - K_2) X.$$

For the chelation equilibrium we have

$$\text{(V)} \qquad X = K_c m (1 - X) \text{ or } X = \frac{K_c m}{1 + K_c m}; \; 1 - X = \frac{1}{1 + K_c m}.$$

Substituting this into IV a and b gives

$$\text{(VI a)} \qquad K = K_1 - \frac{K_1 - K_2}{1 + K_c m},$$

$$\text{(VI b)} \qquad K = K_2 + \frac{m K_c (K_1 - K_2)}{1 + K_c m}.$$

For a kinetic study it is of interest to see in what way an increase in the concentration of the cation will affect the velocity of the reaction. It can be seen that if $K_1 - K_2$ is small, the effect of an increase in the concentration of the cation will be small. It will be small, too, for very low and very high K_c values. This means that the effect of added cations will be small for mainly chelate and mainly ionic reactions as well as those in which the chelate and the ionic reactions have almost the same velocity. It can further be seen that K increases with increasing concentration of the cation if $K_1 - K_2 > 0$, but decreases if $K_1 - K_2 < 0$. These effects may, however, be masked by other salt effects.

The aforegoing considerations have been tested by a kinetic study of the methylation of ethyl acetoacetate in *tert*-butanol and of methyl acetoacetate in methanol.

In *tert*-butanol the methylation reaction was found to be mainly chelate, as, if

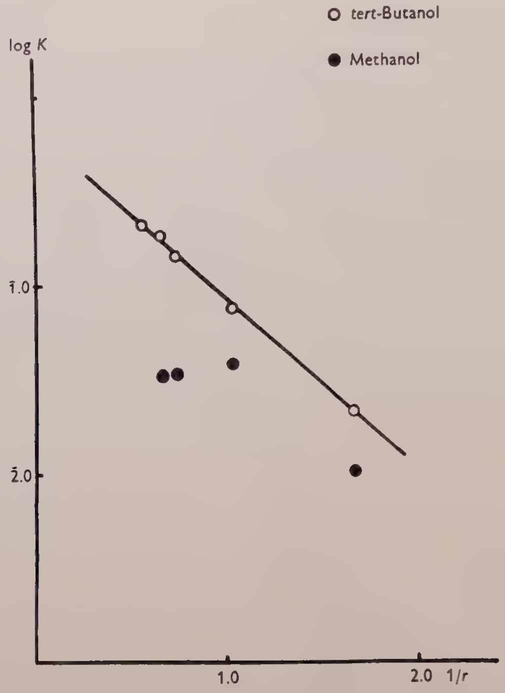


Fig. 1.

different alkali compounds were used (Tables 1–5), a plot of log K against $1/r$ gave a straight line (Fig. 1), indicating that K was very nearly equal to K_1. This result conforms well to the considerations above, where it was pointed out that the use of a solvent of low polarity will favour the chelate reaction.

In a solvent of higher polarity, such as methanol, the chelate mechanism will be much less favoured than in *tert*-butanol. If this decrease in the extent of the chelate

Table 1. The reaction of the lithium compound of ethyl acetoacetate with methyl iodide in *tert*-butanol at 25.00°.

min.	a	b	log a/b
0	0.2036	0.0989	0.3136
15	0.1985	0.0938	0.3256
30	0.1943	0.0896	0.3362
60	0.1841	0.0794	0.3653
90	0.1763	0.0716	0.3914
120	0.1686	0.0639	0.4214
155	0.1620	0.0573	0.4513
181	0.1570	0.0523	0.4774
210	0.1518	0.0471	0.5083
235	0.1488	0.0441	0.5282
$K = 2.05 \cdot 10^{-2}$			

Table 2. The reaction of the sodium compound of ethyl acetoacetate with methyl iodide in *tert*-butanol at 25.00°.

min.	a	b	log a/b
0	0.2036	0.0929	0.3408
15	0.1845	0.0738	0.3779
30	0.1713	0.0606	0.4513
45	0.1622	0.0515	0.4983
60	0.1542	0.0435	0.5496
76	0.1475	0.0368	0.6030
90	0.1425	0.0318	0.6514
105	0.1380	0.0273	0.7037
$K = 7.25 \cdot 10^{-2}$			

Table 3. The reaction of the potassium compound of ethyl acetoacetate with methyl iodide in *tert*-butanol at 25.00°.

min.	a	b	$\log a/b$
0	0.2090	0.0988	0.3254
10	0.1868	0.0766	0.3872
20	0.1693	0.0591	0.4571
30	0.1582	0.0480	0.5180
40	0.1482	0.0380	0.5911
50	0.1420	0.0318	0.6499
60	0.1366	0.0264	0.7139
70	0.1320	0.0218	0.7821
80	0.1282	0.0180	0.8526

$K = 1.37 \cdot 10^{-1}$

Table 4. The reaction of the rubidium compound of ethyl acetoacetate with methyl iodide in *tert*-butanol at 25.00°.

min.	a	b	$\log a/b$
0	0.2090	0.0988	0.3254
7.5	0.1856	0.0754	0.3912
15	0.1686	0.0584	0.4605
22.5	0.1579	0.0477	0.5199
30	0.1485	0.0383	0.5885
37.5	0.1420	0.0318	0.6499
45	0.1366	0.0264	0.7139
52.5	0.1321	0.0219	0.7804

$K = 1.80 \cdot 10^{-1}$

Table 5. The reaction of the cesium compound of ethyl acetoacetate with methyl iodide in *tert*-butanol at 25.00°.

min.	a	b	$\log a/b$
0	0.2090	0.0963	0.3366
7.5	0.1834	0.0707	0.4140
15	0.1656	0.0529	0.4956
22.5	0.1537	0.0410	0.5739
30	0.1452	0.0325	0.6501
37.5	0.1391	0.0264	0.7217
45	0.1341	0.0214	0.7970
52.5	0.1306	0.0179	0.8630

$K = 2.06 \cdot 10^{-1}$

Table 6. The reaction of the lithium compound of methyl acetoacetate with methyl iodide in methanol at 25.00°.

min.	a	b	$\log a/b$
0	0.2004	0.1011	0.2971
60	0.1892	0.0899	0.3231
120	0.1795	0.0802	0.3499
180	0.1719	0.0726	0.3744
240	0.1647	0.0654	0.4011
300	0.1587	0.0594	0.4268
360	0.1537	0.0544	0.4511
420	0.1492	0.0499	0.4757

$K = 0.99 \cdot 10^{-2}$

Table 7. The reaction of the sodium compound of methyl acetoacetate with methyl iodide in methanol at 25.00°.

min.	a	b	$\log a/b$
0	0.2000	0.1008	0.2975
30	0.1819	0.0827	0.3423
60	0.1677	0.0685	0.3888
90	0.1562	0.0570	0.4378
120	0.1477	0.0485	0.4837
150	0.1399	0.0407	0.5362
180	0.1342	0.0350	0.5837
210	0.1297	0.0305	0.6286

$K = 3.70 \cdot 10^{-2}$

Table 8. The reaction of the sodium compound of methyl acetoacetate with methyl iodide in a 0.1 normal methanolic sodium iodide solution at 25.00°.

min.	a	b	$\log a/b$
0	0.2000	0.1005	0.2988
30	0.1814	0.0819	0.3453
60	0.1667	0.0672	0.3945
90	0.1549	0.0554	0.4466
120	0.1462	0.0467	0.4957
150	0.1390	0.0395	0.5464
180	0.1334	0.0339	0.5950
210	0.1282	0.0287	0.6500

$K = 3.87 \cdot 10^{-2}$

Table 9. The reaction of the sodium compound of methyl acetoacetate with methyl iodide in a 0.2 normal methanolic sodium iodide solution at 25.00°.

min.	a	b	log a/b
0	0.2000	0.1005	0.2988
30	0.1805	0.0810	0.3480
60	0.1654	0.0659	0.3996
90	0.1534	0.0539	0.4542
120	0.1445	0.0450	0.5067
150	0.1372	0.0377	0.5611
180	0.1311	0.0316	0.6179
210	0.1267	0.0272	0.6682
$K = 4.11 \cdot 10^{-2}$			

Table 10. The reaction of the potassium compound of methyl acetoacetate with methyl iodide in methanol at 25.00°.

min.	a	b	log a/b
0	0.2004	0.1014	0.2959
30	0.1843	0.0853	0.3346
60	0.1704	0.0714	0.3778
90	0.1605	0.0615	0.4166
120	0.1510	0.0520	0.4630
150	0.1440	0.0450	0.5052
180	0.1376	0.0386	0.5520
210	0.1331	0.0341	0.5914
240	0.1287	0.0297	0.6368
270	0.1251	0.0261	0.6807
$K = 3.34 \cdot 10^{-2}$			

Table 11. The reaction of the potassium compound of methyl acetoacetate with methyl iodide in a 0.1 normal methanolic potassium iodide solution at 25.00°.

min.	a	b	log a/b
0	0.2000	0.1018	0.2932
30	0.1820	0.0838	0.3369
60	0.1674	0.0692	0.3837
90	0.1572	0.0590	0.4256
120	0.1484	0.0502	0.4707
150	0.1415	0.0433	0.5143
180	0.1355	0.0373	0.5602
210	0.1308	0.0326	0.6034
$K = 3.46 \cdot 10^{-2}$			

Table 12. The reaction of the rubidium compound of methyl acetoacetate with methyl iodide in methanol at 25.00°.

min.	a	b	log a/b
0	0.2004	0.1098	0.2613
30	0.1835	0.0929	0.2956
60	0.1699	0.0793	0.3309
90	0.1584	0.0678	0.3686
120	0.1485	0.0579	0.4090
150	0.1413	0.0507	0.4451
180	0.1345	0.0439	0.4862
210	0.1292	0.0386	0.5247
$K = 3.22 \cdot 10^{-2}$			

reaction is sufficiently high, we can expect that the reaction for high values of the ionic radius of the cation will be mainly ionic. This, indeed, was found to be the case, as is evident from a plot of log K against $1/r$ (Fig. 1). In this case the reaction velocity was nearly the same for the sodium, potassium, rubidium, and tetramethylammonium compound, whereas that for the lithium compound was much less (Tables 6–13).

Closer study of the velocity constants reveals that the velocity of the sodium compound was somewhat higher than those for all other cations examined. The existence of this maximum velocity for the sodium compound (quite beyond the experimental error) is very interesting. From equations VI a and VI b it will be seen that K always has a value between K_1 and K_2. For a reaction which is mainly ionic for high values of the ionic radius, K is equal to K_2 at $r = \infty$ and at a value of r where $K_1 = K_2$. Since K is greater than K_2 between these two values, the reaction velocity must have

Table 13. The reaction of the tetramethylammonium compound of methyl acetoacetate with methyl iodide in methanol at 25.00°.

min.	a	b	log a/b
0	0.2004	0.1017	0.2946
30	0.1821	0.0834	0.3391
60	0.1690	0.0703	0.3809
90	0.1579	0.0592	0.4261
120	0.1495	0.0508	0.4687
150	0.1423	0.0436	0.5137
180	0.1363	0.0376	0.5593
210	0.1314	0.0327	0.6041
240	0.1272	0.0285	0.6497
270	0.1236	0.0249	0.6958
$K = 3.46 \cdot 10^{-2}$			

a maximum in this region. The maximum is therefore to be found where $K_1 - K_2 > 0$. Hence, an increase in the concentration of the cation will increase the velocity of the reaction for a cation where this maximum is found. This proved to be the case when sodium iodide was added to a reaction mixture consisting of sodium methyl acetoacetate and methyl iodide in methanol (Tables 7–9). It may be pointed out that the addition of sodium iodide in the reaction of sodium ethoxide and methyl iodide in ethanol caused a decrease in the reaction velocity (4).

Experimental

The ethyl acetoacetate, methyl acetoacetate and methyl iodide were commercial products. The *tert*-butanol was a commercial anhydrous product which was dried by distillation from potassium just before use. The methanol was a commercial product with a very low water content.

The reaction mixtures were kept at 25.00° and were 0.1 normal with respect to the alkali compound and 0.2 normal with respect to the methyl iodide. They were prepared by mixing solutions of the alkoxide, the ketoester and the methyl iodide, in that order. The time was counted from the moment when the methyl iodide had been added.

The alkoxide solutions were prepared by dissolving the alkali metal in the alcohol, but in some cases this was not possible and other methods were used.

The reaction of lithium with *tert*-butanol is very slow, so that the lithium ethyl acetoacetate solution was prepared by mixing solutions of potassium ethyl acetoacetate and lithium chloride and decanting the resulting *tert*-butanol solution of lithium ethyl acetoacetate from the potassium chloride precipitated.

The solution of tetramethylammonium methoxide was similarly prepared from tetramethylammonium chloride and potassium methoxide. Tetramethylammonium *tert*-butoxide could not be prepared by this method since the chloride is not soluble in *tert*-butanol.

The cesium compound was omitted in the measurements in methanol due to experimental difficulties in the preparation of the cesium methoxide solution and since no significant difference could be expected in the alkylation velocity of the cesium and the rubidium compound in this solvent.

The reaction was followed by pipetting out 2 ml portions at fixed intervals and pouring them into 25 ml portions of 0.01-*N* hydrochloric acid. The acid in excess was then titrated by means of 0.05-*N* sodium hydroxide, using methyl red as an indicator.

Summary

The methylation of ethyl acetoacetate in *tert*-butanol and of methyl acetoacetate in methanol was studied, using different alkali derivatives of the esters. The existence of a chelate and an ionic alkylation mechanism was demonstrated, and the factors determining the relative extent of these two reactions were examined.

The author is indebted to Messrs. Pharmacia Ltd. for permission to publish this paper and to Fil. Mag. SOLVEIG HANSEN for assistance with the experimental work.

Research Laboratory of Pharmacia Ltd., Uppsala, January 1954.

REFERENCES

1. BRÄNDSTRÖM, A., Arkiv f. Kemi *6*, 155 (1953).
2. VAN UITERT, LE GRAND G., FERNELIUS, W. C., and DOUGLAS, B. E., J. Am. Chem. Soc. 75, 457 (1953).
3. MARTELL, A. E., and CALVIN, M., Chemistry of the metal chelate compounds. Prentice-Hall, Inc. New York 1952, p. 191.
4. ROBERTSON, H. C., Jr., and ACREE, S. F., J. Am. Chem. Soc. *37*, 1902 (1915).

Tryckt den 6 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 12

Communicated 13 January 1954 by Arne Fredga

The acidolysis of ethyl diacetoacetate

III. The importance of variation of the alcohol component of diacetoacetates in the magnesium oxide catalysed reaction with acetic acid

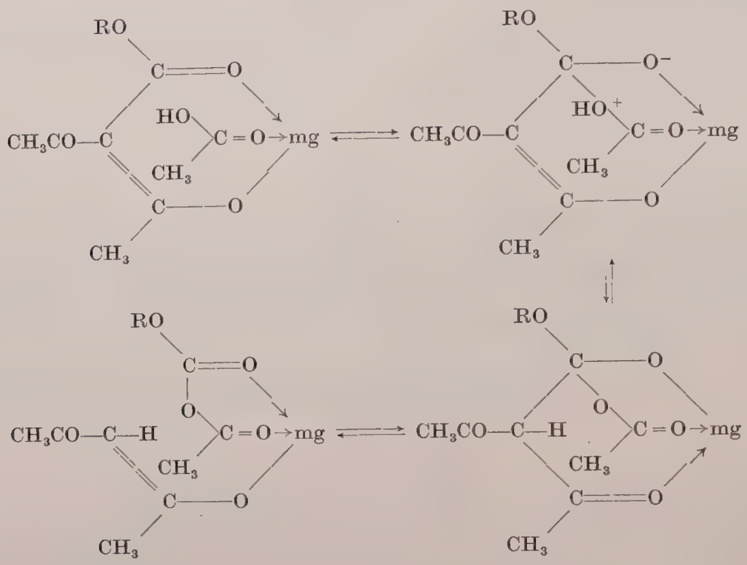
By Arne Brändström

In the first paper in this series (1) the steric possibilities for the acidolysis of ethyl diacetoacetate were considered without further reference to the exact electronic mechanism. An attempt to elucidate this will be made in the present and future investigations.

The most important finding reported in the first paper was that the catalyst must be of such a type as to permit the coordination of both ethyl diacetoacetate and acetic acid to the same metal ion. Since the acidolysis must be a carbonyl reaction associated with the ester group, it is of interest to see in what way a variation of the alcohol component of the ester will affect the velocity of the reaction. Theoretically it can be concluded that the principal reactions may be two, both of the second order. In one of them the acetic acid attacks the chelate of the ester, and in the other the acetate anion attacks the free ester. The question as to which mechanism is the actual one is not concerned solely with the position of a proton, as it may, at first sight, appear to be.

From a comparison of the acid catalysed hydrolysis of esters by the $A_{AC}2$ mechanism (2) and the base catalysed hydrolysis by the $B_{AC}2$ one (2), it can be concluded that an attack on an ester by an anion is far more subject to changes in velocity, by a variation of the alcohol component of the ester, than is an attack by an uncharged molecule. In experiments with the methyl, ethyl, and *iso*propyl esters of diacetoacetic acid, the relative reactivities with glacial acetic acid and magnesium oxide as catalyst were found to be 1 : 0.78 : 0.50; whereas those for the $A_{AC}2$ reaction of the corresponding acetates are 1 : 0.97 : 0.53, and those for the $B_{AC}2$ reaction 1 : 0.60 : 0.146. From this it can be concluded that the acidolysis of ethyl diacetoacetate by acetic acid, with magnesium oxide as the catalyst, probably is a reaction of the magnesium chelate with acetic acid. The reaction can then be formulated in the way shown on the next page (mg represents one equivalent of magnesium):

The mixed anhydride of the acetic acid and the monoalkyl carbonate formed is unstable under the reaction conditions and rapidly decomposes into carbon dioxide and an alkyl acetate. A comparison with the $A_{AC}2$ reaction suggests that the intermediates in the reaction probably are distinct molecules having a life long enough for a proton shift to occur (3).



Experimental

The kinetic measurements were performed in the same way as described previously (1). The results are given in Tables 1–10, where K^* is the reaction constant calculated for the addition of 1 m.mole of the catalyst to a reaction mixture consisting of 0.1 mole of the ester and 0.2 moles of glacial acetic acid.

Summary

The reaction of acetic acid with methyl, ethyl and *iso*propyl diacetoacetate, using magnesium oxide as a catalyst, was found to consist of an attack on the magnesium chelate of the ester by acetic acid.

ACKNOWLEDGEMENTS

The author is indebted to Messrs. Pharmacia Ltd. for permission to publish this paper and to Fil. Mag. Solveig Hansen for valuable help with the experimental work.

Research Laboratory, Pharmacia Ltd., Uppsala, Sweden.

REFERENCES

1. Brändström, A., Arkiv f. Kemi *6* No. 16 (1953).
2. Ingold, C. K., Structure and Mechanism in Organic Chemistry. London 1953, p. 752—782.
3. Bender, M. L., J. Am. Chem. Soc. *73*, 1626 (1951).

Table 1. The reaction of methyl diacetoacetate 0.1 mole, acetic acid 0.2 moles, and magnesium oxide 0.2649 m.moles. $t = 120°$

Min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
15	70	0.0093	0.6574	0.3240	0.3072
30	148	0.0196	0.6471	0.3137	0.3145
45	221	0.0292	0.6375	0.3041	0.3215
60	293	0.0387	0.6280	0.2946	0.3288
75	371	0.0491	0.6176	0.2842	0.3371
90	440	0.0582	0.6085	0.2751	0.3448
105	506	0.0669	0.5998	0.2664	0.3525
120	569	0.0752	0.5915	0.2581	0.3602
135	627	0.0827	0.5840	0.2506	0.3674
150	682	0.0903	0.5764	0.2430	0.3751
165	738	0.0976	0.5691	0.2357	0.3828
180	790	0.1045	0.5622	0.2288	0.3904
195	838	0.1108	0.5559	0.2225	0.3977

$V_\infty = 2519$ ml. $K = 3.48 \cdot 10^{-3}$

Table 2. The reaction of methyl diacetoacetate 0.1 mole, acetic acid 0.2 moles, and magnesium oxide 0.491 m.moles. $t = 120°$

Min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
10	67	0.0090	0.6577	0.3243	0.3070
20	134	0.0179	0.6488	0.3154	0.3154
30	200	0.0267	0.6400	0.3066	0.3196
40	265	0.0354	0.6313	0.2979	0.3261
50	337	0.0450	0.6217	0.2883	0.3338
60	400	0.0534	0.6133	0.2799	0.3407
70	464	0.0619	0.6048	0.2714	0.3480
80	523	0.0698	0.5969	0.2635	0.3551
90	579	0.0773	0.5894	0.2560	0.3622
100	632	0.0844	0.5823	0.2489	0.3692
110	683	0.0912	0.5755	0.2421	0.3761
120	732	0.0977	0.5690	0.2356	0.3829
130	780	0.1041	0.5626	0.2292	0.3900
140	822	0.1098	0.5569	0.2235	0.3965
150	864	0.1153	0.5514	0.2180	0.4030

$V_\infty = 2498$ ml. $K = 4.87 \cdot 10^{-3}$

Table 3. The reaction of methyl diacetoacetate 0.1 mole, acetic acid 0.2 moles, and magnesium oxide 1.029 m.moles. $t = 120^\circ$

Min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0			0.6667	0.3333	0.3010
10	91	0.0120	0.6547	0.3213	0.3091
20	189	0.0250	0.6417	0.3083	0.3183
30	292	0.0386	0.6281	0.2947	0.3286
40	393	0.0519	0.6148	0.2814	0.3394
50	484	0.0640	0.6027	0.2693	0.3499
60	570	0.0753	0.5914	0.2580	0.3603
70	649	0.0858	0.5809	0.2475	0.3705
80	722	0.0954	0.5713	0.2379	0.3805
90	799	0.1045	0.5622	0.2288	0.3904
100	855	0.1131	0.5536	0.2202	0.4004

$V_\infty = 2519$ ml. $K = 7.01 \cdot 10^{-3}$

Table 4. The reaction of methyl diacetoacetate 0.1 mole, acetic acid 0.2 moles, and magnesium oxide 1.607 m.moles. $t = 120^\circ$

Min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0			0.6667	0.3333	0.3010
5	68	0.0091	0.6576	0.3242	0.3072
10	128	0.0171	0.6496	0.3162	0.3127
15	188	0.0251	0.6416	0.3082	0.3185
20	250	0.0334	0.6333	0.2999	0.3246
25	314	0.0419	0.6248	0.2914	0.3312
30	374	0.0501	0.6166	0.2832	0.3379
35	431	0.0577	0.6090	0.2756	0.3443
40	483	0.0647	0.6020	0.2686	0.3505
45	538	0.0720	0.5947	0.2613	0.3572
50	588	0.0786	0.5881	0.2547	0.3635
55	636	0.0851	0.5816	0.2482	0.3698
60	681	0.0912	0.5755	0.2421	0.3761
65	722	0.0967	0.5700	0.2366	0.3819

$V_\infty = 2491$ ml. $K = 8.65 \cdot 10^{-3}$

Table 5. The reaction of methyl diacetoacetate 0.1 mole with acetic acid 0.2 moles using various amounts of magnesium oxide. $t = 120^\circ$

m.mol MgO	$\sqrt{\text{m.mol MgO}}$	$K\ 10^3$	$K^*\ 10^3$
0.2649	0.515	3.48	6.76
0.491	0.701	4.87	6.95
1.029	1.014	7.01	6.91
1.607	1.268	8.65	6.82

$K^* = 6.86 \cdot 10^{-3}$

Table 6. The reaction of ethyl diacetoacetate 0.1 mole, with acetic acid 0.2 moles using various amounts of magnesium oxide. $t = 120°$

m.mol MgO	$\sqrt{\text{m.mol MgO}}$	$K \cdot 10^3$	$K^* \cdot 10^3$
0.0595	0.244	1.21	4.96
0.2225	0.472	2.50	5.30
0.4899	0.699	3.75	5.36
1.094	1.046	5.82	5.57
1.762	1.327	7.14	5.38

$K^* = 5.32 \cdot 10^{-3}$

Table 7. The reaction of *iso*propyl diacetoacetate 0.1 mole, acetic acid 0.2 moles, and magnesium oxide 0.271 m.moles. $t = 120°$

Min.	ml CO_2	x	$a - x$	$b - x$	$\log \frac{a - x}{b - x}$
0			0.6667	0.3333	0.3010
30	100	0.0133	0.6534	0.3200	0.3100
60	180	0.0239	0.6428	0.3094	0.3176
90	256	0.0339	0.6328	0.2994	0.3250
120	317	0.0421	0.6246	0.2912	0.3314
150	381	0.0506	0.6161	0.2827	0.3384
180	455	0.0603	0.6064	0.2730	0.3466
210	525	0.0696	0.5971	0.2637	0.3550
240	597	0.0792	0.5975	0.2541	0.3640
270	669	0.0887	0.5780	0.2446	0.3734
300	731	0.0969	0.5698	0.2364	0.3820
330	789	0.1048	0.5619	0.2285	0.3905
360	853	0.1131	0.5536	0.2202	0.4004
390	909	0.1207	0.5460	0.2126	0.4096
420	965	0.1280	0.5387	0.2053	0.4190

$V_\infty = 2515$ ml. $K = 1.88 \cdot 10^{-3}$

Table 8. The reaction of *iso*propyl diacetoacetate 0.1 mole, acetic acid 0.2 moles, and magnesium oxide 0.898 m.moles. $t = 120°$

Min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0			0.6667	0.3333	0.3010
30	160	0.0222	0.6445	0.3111	0.3163
60	282	0.0392	0.6275	0.2941	0.3291
90	398	0.0553	0.6114	0.2780	0.3423
120	508	0.0707	0.5960	0.2626	0.3560
150	612	0.0849	0.5818	0.2484	0.3696
180	709	0.0984	0.5683	0.2349	0.3837
210	795	0.1103	0.5564	0.2230	0.3971
240	873	0.1212	0.5455	0.2121	0.4103
270	949	0.1317	0.5350	0.2016	0.4239
300	1023	0.1419	0.5248	0.1914	0.4381
330	1096	0.1521	0.5146	0.1812	0.4533
360	1172	0.1626	0.5041	0.1707	0.4703
390	1233	0.1713	0.4954	0.1620	0.4855

$V_\infty = 2400$ ml. $K = 3.14 \cdot 10^{-3}$

Table 9. The reaction of *iso*propyl diacetoacetate 0.1 mole, acetic acid 0.2 moles, and magnesium oxide 1.488 m.moles. $t = 120°$

Min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0			0.6667	0.3333	0.3010
15	118	0.0158	0.6509	0.3175	0.3118
30	217	0.0288	0.6379	0.3045	0.3212
45	297	0.0394	0.6273	0.2939	0.3293
60	379	0.0503	0.6164	0.2830	0.3381
75	453	0.0600	0.6067	0.2733	0.3464
90	528	0.0699	0.5968	0.2634	0.3552
105	601	0.0797	0.5870	0.2536	0.3644
120	672	0.0890	0.5777	0.2443	0.3738
135	739	0.0978	0.5689	0.2355	0.3830
150	800	0.1059	0.5608	0.2274	0.3920
165	861	0.1140	0.5527	0.2193	0.4015
180	920	0.1219	0.5448	0.2114	0.4111

$V_\infty = 2517$ ml. $K = 4.20 \cdot 10^{-3}$

Table 10. The reaction of *iso*propyl diacetoacetate 0.1 mole with acetic acid 0.2 moles using various amounts of magnesium oxide. $t = 120°$

m.mol MgO	$\sqrt{\text{m.mol MgO}}$	$K\ 10^3$	$K^*\ 10^3$
0.271	0.521	1.88	3.61
0.898	0.948	3.14	3.31
1.488	1.220	4.20	3.44

$K^* = 3.45 \cdot 10^{-3}$

Tryckt den 6 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 13

Communicated 10 February 1954 by Arne Fredga

The acidolysis of ethyl diacetoacetate

IV. Effect of variation of the alcohol component of alkyl diacetoacetates on the velocity of the reaction catalysed by sodium acetate

By Arne Brändström

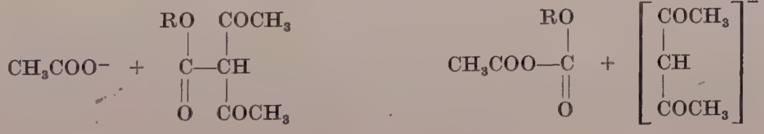
In a recent paper (1) the writer investigated the reaction of ethyl diacetoacetate with acetic acid catalysed by magnesium oxide and found that it could be considered as a reaction of the magnesium chelate with the acetic acid.

If an acetate containing a cation with only a slight tendency to form chelate compounds is used as a catalyst, the reaction mechanism might be expected to change into one classifiable as a reaction of the ester with the acetate anion.

As pointed out in the previous paper, these two reactions are affected to a different extent by a variation of the alcohol component of the ester. For the magnesium oxide catalysed reaction the relative velocity coefficients of the methyl, ethyl and *iso*propyl esters were 1 : 0.78 : 0.50, which values closely accord with the acid catalysed hydrolysis of the corresponding acetates (1 : 0.97 : 0.53). For the sodium acetate catalysed reaction the corresponding relative velocity coefficients were found to be 1 : 0.65 : 0.31, and hence the mechanism is different from that in the reaction catalysed by magnesium oxide.

In a reaction which consists in an attack on an ester by an uncharged anionoid reagent, the effect of a variation of the alcohol component is mainly steric (2), wherefore we may expect to find the same relative velocity coefficients for most reactions of this type if the components are not too bulky. This would explain the fairly close agreement between the magnesium oxide catalysed reaction and the acid catalysed hydrolysis of the acetate.

In a reaction which consists of an attack on an ester by a negatively charged anionoid reagent, the effect of a variation of the alcohol component is due to both steric and electronic influences. Since the sodium acetate catalysed reaction mechanism differs from that in the case of magnesium oxide, it is probably of this type and can be formulated as



$$CH_3COO^- + \begin{array}{c} RO \\ | \\ C \\ \| \\ O \end{array}\!-\!\begin{array}{c} COCH_3 \\ | \\ CH \\ | \\ COCH_3 \end{array} \qquad CH_3COO-\begin{array}{c} RO \\ | \\ C \\ \| \\ O \end{array} + \left[\begin{array}{c} COCH_3 \\ | \\ CH \\ | \\ COCH_3 \end{array}\right]^-$$

Table 1. The reaction of methyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120°.

min.	ml CO_2	x	$b-x$	$\log b-x$
0	0		0.3333	$\bar{1}.5228$
30	40	0.0053	0.3280	$\bar{1}.5159$
60	89	0.0118	0.3215	$\bar{1}.5072$
90	146	0.0194	0.3139	$\bar{1}.4968$
120	200	0.0266	0.3067	$\bar{1}.4867$
150	258	0.0343	0.2990	$\bar{1}.4757$
180	316	0.0420	0.2913	$\bar{1}.4643$
210	374	0.0497	0.2836	$\bar{1}.4527$
240	432	0.0575	0.2758	$\bar{1}.4406$
270	484	0.0643	0.2690	$\bar{1}.4298$
300	536	0.0713	0.2620	$\bar{1}.4183$
330	587	0.0781	0.2552	$\bar{1}.4069$
360	640	0.0852	0.2481	$\bar{1}.3946$
390	691	0.0920	0.2413	$\bar{1}.3826$

301.7 mg Na_2CO_3 $V_\infty = 2508$ ml $K = 8.75 \cdot 10^{-4}$

Table 2. The reaction of methyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120°.

min.	ml CO_2	x	$b-x$	$\log b-x$
0	0		0.3333	$\bar{1}.5228$
20	55	0.0074	0.3259	$\bar{1}.5131$
40	105	0.0142	0.3191	$\bar{1}.5039$
60	157	0.0212	0.3121	$\bar{1}.4943$
80	213	0.0287	0.3046	$\bar{1}.4837$
100	268	0.0362	0.2971	$\bar{1}.4729$
120	329	0.0444	0.2889	$\bar{1}.4608$
140	385	0.0520	0.2813	$\bar{1}.4492$
160	440	0.0594	0.2739	$\bar{1}.4376$
180	496	0.0670	0.2663	$\bar{1}.4254$
200	548	0.0740	0.2593	$\bar{1}.4138$
220	598	0.0807	0.2526	$\bar{1}.4024$
240	648	0.0875	0.2458	$\bar{1}.3906$
260	696	0.0939	0.2394	$\bar{1}.3791$
280	740	0.0999	0.2334	$\bar{1}.3681$
300	783	0.1057	0.2276	$\bar{1}.3572$

500.1 mg Na_2CO_3 $V_\infty = 2470$ ml $K = 13.3 \cdot 10^{-4}$

Table 3. The reaction of methyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120°.

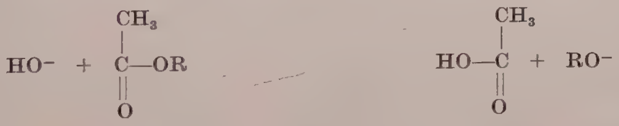
min.	ml CO_2	x	$b-x$	$\log b-x$
0	0		0.3333	$\bar{1}.5228$
20	46	0.0062	0.3271	$\bar{1}.5147$
40	114	0.0154	0.3179	$\bar{1}.5023$
60	193	0.0262	0.3071	$\bar{1}.4873$
80	281	0.0381	0.2952	$\bar{1}.4701$
100	364	0.0494	0.2839	$\bar{1}.4532$
120	440	0.0597	0.2736	$\bar{1}.4371$
140	512	0.0695	0.2638	$\bar{1}.4213$
160	583	0.0792	0.2541	$\bar{1}.4050$
180	650	0.0883	0.2450	$\bar{1}.3892$
200	713	0.0967	0.2366	$\bar{1}.3740$
220	775	0.1052	0.2281	$\bar{1}.3581$

750.5 mg Na_2CO_3 $V_\infty = 2456$ ml $K = 18.5 \cdot 10^{-4}$

Table 4. The reaction of methyl diacetoacetate with glacial acetic acid using different amounts of sodium carbonate as a catalyst.

mg Na_2CO_3	m.equ. Na_2CO_3	(m.equ. $Na_2CO_3)^{3/4}$	$K \cdot 10^4$	$K^* \cdot 10^4$
301.7	5.69	3.69	8.75	2.37
500.1	9.44	5.38	13.3	2.47
750.5	14.16	7.30	18.5	2.53

If it is compared with the base catalysed hydrolysis of alkyl acetates



$$HO^- + CH_3-C(=O)-OR \longrightarrow HO-C(CH_3)(=O) + RO^-$$

it will be seen that the charge of the anionoid reagent is transferred to different atoms in these two reactions. In the hydrolysis reaction only, it is transferred to the alcohol residue. It may accordingly be expected that the velocity of this reaction will be more dependent on the alcohol component than will the sodium acetate catalysed acidolysis of alkyl diacetoacetates. The difference in the observed relative reaction velocities for the methyl, ethyl and *iso*propyl esters in these reactions—i.e., 1 : 0.601 : 0.146 and 1 : 0.65 : 0.31—may well accord with this.

The velocity of the sodium acetate catalysed acidolysis is, as far as can be concluded from the experimental results, independent of the concentration of

Table 5. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120°.

min.	ml CO_2	x	$b-x$	$\log b-x$
0	0		0.3333	$\bar{1}.5228$
30	36	0.0049	0.3284	$\bar{1}.5164$
60	75	0.0102	0.3231	$\bar{1}.5093$
90	118	0.0161	0.3172	$\bar{1}.5013$
120	163	0.0222	0.3111	$\bar{1}.4929$
150	206	0.0281	0.3052	$\bar{1}.4846$
180	254	0.0346	0.2987	$\bar{1}.4752$
210	300	0.0410	0.2923	$\bar{1}.4658$
240	346	0.0472	0.2861	$\bar{1}.4565$
270	389	0.0530	0.2803	$\bar{1}.4476$
300	434	0.0592	0.2741	$\bar{1}.4379$
330	477	0.0651	0.2682	$\bar{1}.4285$
360	522	0.0712	0.2621	$\bar{1}.4185$
390	567	0.0774	0.2559	$\bar{1}.4081$
420	608	0.0830	0.2503	$\bar{1}.3985$

410.8 mg Na_2CO_3 $V_\infty = 2443$ $K = 7.21 \cdot 10^{-4}$

Table 6. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120°.

min.	ml CO_2	x	$b-x$	$\log b-x$
0	0		0.3333	$\bar{1}.5228$
30	34	0.0046	0.3287	$\bar{1}.5168$
60	92	0.0124	0.3209	$\bar{1}.5064$
90	151	0.0204	0.3129	$\bar{1}.4954$
120	214	0.0288	0.3045	$\bar{1}.4836$
150	278	0.0374	0.2959	$\bar{1}.4711$
180	341	0.0459	0.2874	$\bar{1}.4585$
210	400	0.0539	0.2794	$\bar{1}.4462$
240	462	0.0623	0.2710	$\bar{1}.4330$
270	528	0.0711	0.2622	$\bar{1}.4186$
300	588	0.0792	0.2541	$\bar{1}.4050$
330	648	0.0873	0.2460	$\bar{1}.3909$
360	707	0.0953	0.2380	$\bar{1}.3766$
390	764	0.1030	0.2303	$\bar{1}.3623$
420	820	0.1105	0.2228	$\bar{1}.3479$
450	872	0.1175	0.2158	$\bar{1}.3341$

650.6 mg Na_2CO_3 $V_\infty = 2474$ $K = 10.5 \cdot 10^{-4}$

Table 7. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120°.

min.	ml CO_2	x	$b-x$	$\log b-x$
0	0		0.3333	$\bar{1}.5228$
30	68	0.0092	0.3241	$\bar{1}.5107$
60	155	0.0209	0.3124	$\bar{1}.4947$
90	246	0.0331	0.3002	$\bar{1}.4774$
120	343	0.0462	0.2871	$\bar{1}.4580$
150	432	0.0582	0.2751	$\bar{1}.4395$
180	522	0.0703	0.2630	$\bar{1}.4200$
210	594	0.0800	0.2533	$\bar{1}.4036$
240	683	0.0920	0.2413	$\bar{1}.3826$
270	743	0.1001	0.2332	$\bar{1}.3677$
300	838	0.1129	0.2204	$\bar{1}.3432$
330	912	0.1229	0.2104	$\bar{1}.3231$

1002 mg Na_2CO_3 $V_\infty = 2474$ $K = 14.6 \cdot 10^{-4}$

Table 8. The reaction of ethyl diacetoacetate with glacial acetic acid using different amounts of sodium carbonate as a catalyst.

mg Na_2CO_3	m.equ. Na_2CO_3	(m.equ. $Na_2CO_3)^{3/4}$	$K \cdot 10^4$	$K^* \cdot 10^4$
410.8	7.75	4.65	7.21	1.55
650.6	12.27	6.56	10.5	1.60
1002	18.91	9.07	14.6	1.61

the acetic acid but proportional to the concentration of the ester and to the three-fourth power of the amount of catalyst added. This result can hardly be explained other than by assuming that the ester (containing three polar groups) is coordinated to the sodium ion (with the most stable coordination number 4), and that this complex reacts with the acetate anion. The significance of this coordination will be the subject of a future paper.

Experimental

The kinetic measurements were performed in the same way as that described in the first paper in this series (3).

At the end of one experiment with the *iso*propyl diacetoacetate a small quantity of bromine was introduced into the gas buret containing the carbon dioxide. Since no discoloration of the bromine vapor occurred, it was concluded that the carbon dioxide was substantially free from propene.

The catalyst was added in the form of anhydrous sodium carbonate.

Table 9. The reaction of *iso*propyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120°.

min.	ml CO_2	x	$b-x$	$\log b-x$
0	0		0.3333	$\bar{1}.5228$
30	32	0.0044	0.3289	$\bar{1}.5171$
60	74	0.0101	0.3232	$\bar{1}.5095$
90	100	0.0136	0.3197	$\bar{1}.5047$
120	130	0.0177	0.3156	$\bar{1}.4991$
150	169	0.0230	0.3103	$\bar{1}.4918$
180	208	0.0283	0.3050	$\bar{1}.4843$
210	248	0.0337	0.2996	$\bar{1}.4765$
240	290	0.0395	0.2938	$\bar{1}.4681$
270	332	0.0452	0.2881	$\bar{1}.4595$
300	372	0.0506	0.2827	$\bar{1}.4513$
330	414	0.0563	0.2770	$\bar{1}.4425$
360	456	0.0620	0.2713	$\bar{1}.4335$
390	499	0.0679	0.2654	$\bar{1}.4239$
420	538	0.0732	0.2601	$\bar{1}.4151$
450	580	0.0789	0.2544	$\bar{1}.4055$

1003.6 mg Na_2CO_3 $V_\infty = 2450$ $K = 6.75 \cdot 10^{-4}$

Table 10. The reaction of *iso*propyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120°.

min.	ml CO_2	x	$b-x$	$\log b-x$
0	0		0.3333	$\bar{1}.5228$
30	43	0.0057	0.3276	$\bar{1}.5153$
60	97	0.0129	0.3204	$\bar{1}.5057$
90	149	0.0198	0.3135	$\bar{1}.4962$
120	190	0.0252	0.3081	$\bar{1}.4887$
150	272	0.0361	0.2972	$\bar{1}.4731$
180	332	0.0441	0.2892	$\bar{1}.4612$
210	392	0.0521	0.2812	$\bar{1}.4490$
240	450	0.0598	0.2735	$\bar{1}.4370$
270	516	0.0685	0.2648	$\bar{1}.4229$
300	572	0.0760	0.2573	$\bar{1}.4104$
330	631	0.0838	0.2495	$\bar{1}.3971$
360	683	0.0908	0.2425	$\bar{1}.3847$
390	742	0.0987	0.2346	$\bar{1}.3703$
420	778	0.1034	0.2299	$\bar{1}.3614$

1532.3 mg Na_2CO_3 $V_\infty = 2508$ $K = 9.62 \cdot 10^{-4}$

Table 11. The reaction of *iso*propyl diacetoacetate with glacial acetic acid using different amounts of sodium carbonate as a catalyst.

mg Na_2CO_3	m.equ. Na_2CO_3	(m.equ. $Na_2CO_3)^{3/4}$	$K \cdot 10^4$	$K^* \cdot 10^4$
1003.6	18.94	9.08	6.75	0.744
1532.3	28.91	12.47	9.62	0.772

Summary

The reaction of methyl, ethyl and *iso*propyl diacetoacetate with acetic acid was studied, using sodium acetate as the catalyst. The reaction velocity was found to be proportional to the concentration of alkyl diacetoacetate and to the three-fourth power of the amount of catalyst added. The relative reaction velocities of the different esters seem to be consistent with a reaction of the ester, probably coordinated to the sodium ion with the acetate anion.

The author is indebted to Messrs. Pharmacia Ltd. for permission to publish this paper and to Fil. Mag. SOLVEIG HANSEN for assistance with the experimental work.

Research Laboratory of Pharmacia Ltd., Uppsala, February 1954.

REFERENCES

(1) BRÄNDSTRÖM, A., Arkiv för Kemi, in press.
(2) INGOLD, C. K., Structure and mechanism in organic chemistry. London 1953, p. 777.
(3) BRÄNDSTRÖM, A., Arkiv för Kemi *6*, nr 16 (1953).

Tryckt den 6 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 14

Communicated 10 February 1954 by ARNE FREDGA and GUNNAR HÄGG

The acidolysis of ethyl diacetoacetate

V. Importance of coordination of the ester to the cation of the catalyst

By ARNE BRÄNDSTRÖM

With 1 figure in the text

The mechanism for the acidolysis of ethyl diacetoacetate in the presence of metal acetates (or other salts of weak acids) has been the subject of a kinetic investigation to determine the role of the metal cation in the reaction. Some results of the investigation have been published in previous papers (1–4). The following types of reactions can be distinguished:

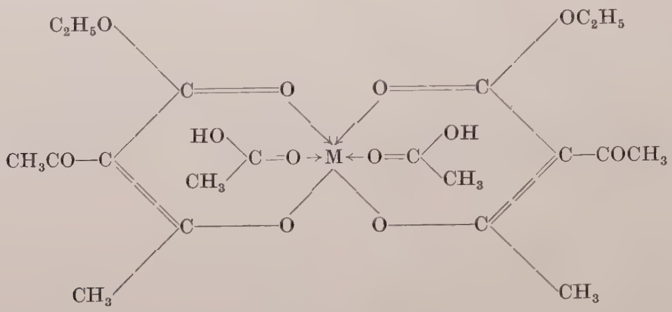
1. A reaction in which the reaction velocity is proportional to the concentration of the acetic acid and ethyl diacetoacetate, and to the square root of the amount of catalyst added.

2. A reaction in which the reaction velocity appears to be proportional to the concentration of ethyl diacetoacetate, and to the three-fourth power of the amount of catalyst added.

The present paper is concerned with the effect of the ionic radius and the stable coordination number of the catalyst on the velocity of the reaction. For this purpose the reaction velocities obtained with a number of different metal catalysts have been measured. As it was necessary to have a stable metal compound of a weak acid (oxide, hydroxide, acetate, carbonate, or acetylacetonate), which is resistent to oxidation or reduction and soluble in the reaction mixture, the number of catalysts available is limited. The fact that metal compounds with a stable coordination number twice the valence have no catalytic effect makes the number of catalysts available for a kinetic investigation still more limited, especially as β-diketones tend to stabilize the oxidation stages with a valence equal to half the stable coordination number. These facts exclude the elements with an atomic number over 30 except those in the first and second group of the Periodic Table. The cations tried are given in Table 1.

The results of the kinetic measurements were in agreement with the two reaction types mentioned above; the reactions catalysed by the bivalent cations were of the first type, whereas those catalysed by the alkali cations were of the second type. In previous papers (3, 4) it has been demonstrated that the reaction catalysed by magnesium oxide is of the first type and in agreement with a mechanism classifiable as a reaction of the metal chelate with acetic acid coordinated to the central cation in the chelate. On the other hand the reaction catalysed by sodium acetate is of the second type and probably in agreement with a reaction of the ester coordinated to the cation with a free acetate anion. The variation of the velocity of these two types of reactions with the ionic radius (r) of the cation in the catalyst has provided further evidence for the mechanisms given.

In a reaction of type 1 catalysed by a bivalent cation with the coordination number 6, the following reactive complex has been assumed:



This type of complex can be considered as formed from a bivalent cation, two moles of the ketoester, and two moles of the acetic acid with the removal of two protons. The actual concentration of this complex will thus be proportional to ma^2b^2 where m, a and b are respectively the concentrations of the cation, the acid and the ester. If this complex is decomposing, forming two moles of carbon dioxide, it can be expected that the velocity of this evolution will be proportional to $ab\sqrt{m}$ in good agreement with the experimental results. In this reaction the acetic acid which enters into the reaction with the metal chelate (3) must be coordinated to the central cation of the chelate. This can be concluded from the fact that the bivalent cations with the characteristic coordination number 4, such as Cu^{2+} and Be^{2+} where this coordination of the acetic acid is impossible, do not catalyse the reaction, whereas the bivalent cations with the characteristic coordination number 6 such as Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} are very good catalysts. The bivalent cations Ni^{2+} and Zn^{2+} which besides the coordination number 4 have the coordination number 6 in many compounds do catalyse the reaction but to a lesser extent than would be expected if they had the coordination number 6 exclusively (Fig. 1).

The bivalent cations mentioned above have a high coordination tendency so that it can be assumed that they exist entirely in the form of chelates. From the magnetic and other properties of chelates of this type it can be concluded that the metal-oxygen bonds in all these compounds are of the ionic type (5). This means that the difference in the catalytic effect of the chelate forming cations is due to the different electrostatic attractions of the cations and the tendency of the chelate for the coordination of the acetic acid. Due to the ionic nature of all bonds with the cations in the solution it can be concluded that all effects, other than those arising from the difference in the coordination number, on the velocity constant K are of such a type that a plot of log K against $1/r$ should give a straight line for all ions with the same coordination number. Such a plot is given in Fig. 1. The fact that the reaction velocity is decreasing with an increasing ionic radius indicates that the electron withdrawing from the ester anion is the most important factor. This is in good agreement with what can be expected from the mechanism given.

The reaction of type 2 catalysed by an alkali acetate is much more difficult to study. This is mainly due to the rather low catalytic effects of these cations which makes it necessary to use high concentrations of the catalyst. A consequence of

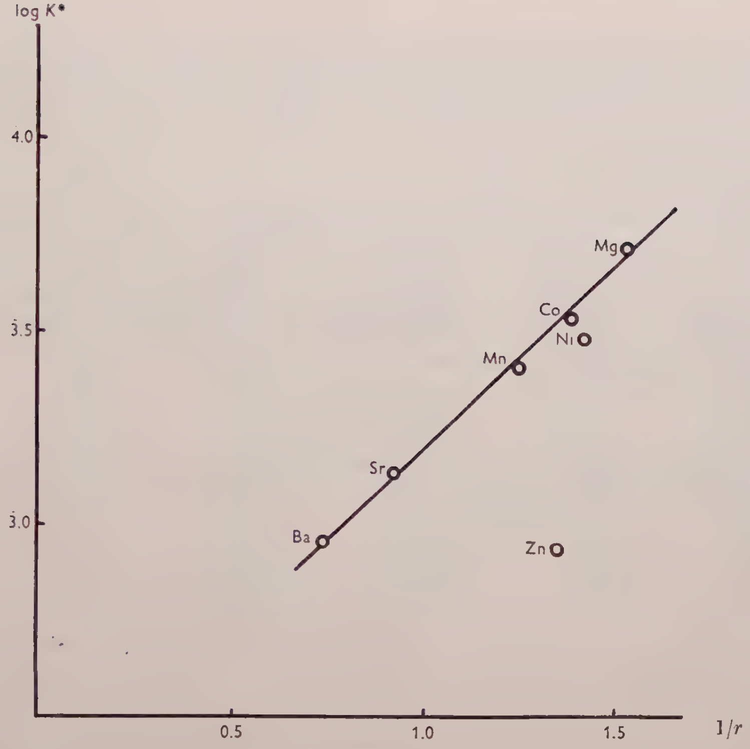


Fig. 1.

this is that impurities in the catalyst are a very important factor. It may be mentioned that 0.6 mg of magnesium oxide has the same catalytic effect as 1 g of rubidium carbonate. Cesium carbonate was not available in such a purity that reproducible results could be obtained.

The high concentration of the catalyst had the further effect that the solutions were far from ideal, with the consequence that the reaction order with respect to the ester and the acetic acid could not be determined with any certainty. In the calculations the reaction velocity has been assumed to be proportional to the concentration of the ester but independent of the concentration of the acetic acid, in agreement with the kinetic result obtained with Li^+ (1).

The reaction velocity was found to decrease rapidly with increasing ionic radius of the alkali cation of the catalyst. The velocity found with tetramethylammonium acetate (high ionic radius) as the catalyst was very low and the real value might be still lower since at least some of the catalytic effect might be due to minute amounts of other catalysts in the tetramethylammonium acetate; *e.g.* 0.01 per cent of magnesium oxide.

The results obtained with different esters of diacetoacetic acid using sodium acetate as the catalyst (4) seem to indicate a reaction of the ester with the acetate ion. The fact that the reaction velocity decreases with increasing ionic radius of the cation in the catalyst indicates further that this cation is important for the reaction. A coordination of the ester to the cation would increase the reactivity of the ester, whereas the coordination of the acetate ion would decrease the anionoid reactivity of this ion. These effects are of increasing importance with decreasing ionic radius of the cation. The results obtained with different cations indicate that only the ester can be coordinated to the cation, whereas the acetate ion entering the reaction must be free.

The results obtained by the kinetic measurements of the acidolysis of ethyl diacetoacetate are very interesting from a more general aspect. The measurements were performed in a very polar reaction medium using rather dilute solutions of many cations, but in no case was a reaction found where the property of the cation was without importance for the reaction. This means that *no pure ionic mechanism was found for the anionoid reaction studied and it can be expected that the same is valid for most anionoid reactions of tautomeric substances.*

Experimental

The kinetic measurements were performed at 120° in the same way as described previously (1), and the results are given in Tables 1–25.

Table 1. Coordination number and catalytic effect.

Cation	Li^+	Be^{2+}	Na^+	Mg^{2+}	Al^{3+}	K^+	Ca^{2+}	Mn^{2+}	Fe^{3+}	Co^{2+}
Coordination number	4	4	4	6	6	4	6	6	6	6
Catalytic effect	+	0	+	+	0	+	+	+	0	+

Cation	Co^{3+}	Ni^{2+}	Cu^{2+}	Zn^{2+}	Rb^+	Sr^{2+}	Cd^{2+}	In^{3+}	Cs^+	Ba^{2+}
Coordination number	6	(4)6	4	4(6)	4(6)	6	4(6)	6	4(6)	6
Catalytic effect	0	+	0	+	+	+	+	0	+	+

Table 2. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using manganous acetate as a catalyst.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	32	0.0043	0.6624	0.3290	0.3039
60	68	0.0092	0.6575	0.3241	0.3072
90	104	0.0140	0.6527	0.3193	0.3105
120	133	0.0179	0.6488	0.3154	0.3132
150	165	0.0222	0.6445	0.3111	0.3163
180	200	0.0269	0.6398	0.3064	0.3197
210	240	0.0323	0.6344	0.3010	0.3238
240	265	0.0357	0.6310	0.2976	0.3264
270	297	0.0400	0.6267	0.2933	0.3298
300	333	0.0449	0.6218	0.2884	0.3337
330	361	0.0486	0.6181	0.2847	0.3367
360	392	0.0528	0.6139	0.2805	0.3402
390	418	0.0563	0.6104	0.2770	0.3431
420	449	0.0605	0.6062	0.2728	0.3468
	15.94 mg $Mn(CH_3COO)_2$		$V_\infty = 2474$ ml		$K = 7.62 \cdot 10^{-4}$

Table 3. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using manganous acetate as a catalyst.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	32	0.0043	0.6624	0.3290	0.3039
60	76	0.0101	0.6566	0.3232	0.3078
90	113	0.0151	0.6516	0.3182	0.3113
120	149	0.0199	0.6468	0.3134	0.3147
150	183	0.0244	0.6423	0.3089	0.3179
180	218	0.0291	0.6376	0.3042	0.3214
210	255	0.0340	0.6327	0.2993	0.3251
240	296	0.0395	0.6272	0.2938	0.3293
270	334	0.0446	0.6221	0.2887	0.3334
300	371	0.0495	0.6172	0.2838	0.3374
330	403	0.0537	0.6130	0.2796	0.3409
360	452	0.0603	0.6064	0.2730	0.3466
390	474	0.0632	0.6035	0.2701	0.3492
420	506	0.0675	0.5992	0.2658	0.3530
450	540	0.0720	0.5947	0.2613	0.3572
480	570	0.0760	0.5907	0.2573	0.3610

20.14 mg $Mn(CH_3COO)_2$ $V_\infty = 2500$ ml $K = 8.90 \cdot 10^{-4}$

Table 4. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using manganous acetate as a catalyst.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	34	0.0046	0.6621	0.3287	0.3041
60	80	0.0108	0.6559	0.3225	0.3083
90	126	0.0170	0.6497	0.3163	0.3126
120	172	0.0232	0.6435	0.3101	0.3171
150	215	0.0290	0.6377	0.3043	0.3213
180	260	0.0351	0.6316	0.2982	0.3259
210	304	0.0410	0.6257	0.2923	0.3306
240	346	0.0467	0.6200	0.2866	0.3351
270	388	0.0524	0.6143	0.2809	0.3398
300	424	0.0572	0.6095	0.2761	0.3439
330	464	0.0626	0.6041	0.2707	0.3486
360	502	0.0677	0.5990	0.2656	0.3532
390	537	0.0725	0.5942	0.2608	0.3576
420	572	0.0772	0.5895	0.2561	0.3621
450	607	0.0819	0.5848	0.2514	0.3666
480	645	0.0870	0.5797	0.2463	0.3717

25.48 mg $Mn(CH_3COO)_2$ $V_\infty = 2470$ ml $K = 10.38 \cdot 10^{-4}$

Table 5. The velocity constant for various amounts of manganous acetate.

mg	m.mol.	$\sqrt{\text{m.mol.}}$	$K \cdot 10^4$	$K^* \cdot 10^3$
15.94	0.0921	0.3025	7.62	2.52
20.14	0.1164	0.3412	8.90	2.61
25.48	0.1473	0.3838	10.38	2.70

Table 6. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using nickel acetate as a catalyst.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	29	0.0039	0.6628	0.3294	0.3037
60	88	0.0118	0.6549	0.3215	0.3090
90	143	0.0192	0.6475	0.3141	0.3141
120	201	0.0270	0.6397	0.3063	0.3198
150	258	0.0347	0.6320	0.2986	0.3256
180	308	0.0414	0.6253	0.2919	0.3309
210	362	0.0486	0.6181	0.2847	0.3367
240	411	0.0552	0.6115	0.2781	0.3422
270	461	0.0619	0.6048	0.2714	0.3480
300	508	0.0682	0.5985	0.2651	0.3537
330	554	0.0744	0.5923	0.2589	0.3594
360	596	0.0801	0.5866	0.2532	0.3648
390	636	0.0855	0.5812	0.2478	0.3702
420	678	0.0911	0.5756	0.2422	0.3759
450	720	0.0967	0.5700	0.2366	0.3819
31.76 mg $Ni(CH_3COO)_2$			$V_\infty = 2481$ ml	$K = 1.28 \cdot 10^{-3}$	

Calcium acetate was found to be too sparingly soluble in the reaction mixture to be used in a kinetic measurement.

Strontium acetate was also sparingly soluble, but supersaturated solutions could be prepared by dissolving the catalyst in the acetic acid and then adding the ethyl diacetoacetate. These solutions were stable enough to permit measurements.

Manganic tris-acetylacetonate was found to behave in the same way as cobaltic tris-acetylacetonate (2), but it was reduced too rapidly for a kinetic investigation.

Summary

The acidolysis of ethyl diacetoacetate has been studied using different catalysts.

The reaction catalysed by compounds containing a bivalent cation with the coordination number 6 was found to be of the same type as that found for the reaction catalysed by magnesium oxide (1, 3).

The reaction catalysed by the alkali acetates was found to be the same as that given when sodium acetate was the catalyst (4).

The velocity of both reactions decreases with increasing ionic radius of the catalyst, indicating that a coordination of the ester to the cation is very important for the reaction.

In no case studied was a pure ionic reaction found.

Table 7. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using nickel acetate as a catalyst.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	52	0.0070	0.6597	0.3263	0.3058
60	126	0.0170	0.6497	0.3163	0.3126
90	199	0.0269	0.6398	0.3064	0.3197
120	272	0.0367	0.6300	0.2966	0.3271
150	341	0.0461	0.6206	0.2872	0.3346
180	411	0.0556	0.6111	0.2777	0.3425
210	474	0.0642	0.6025	0.2691	0.3501
240	530	0.0718	0.5949	0.2615	0.3569
270	587	0.0795	0.5872	0.2538	0.3643
300	641	0.0868	0.5799	0.2465	0.3718
330	695	0.0940	0.5727	0.2393	0.3790
360	732	0.0991	0.5676	0.2342	0.3844
390	789	0.1067	0.5600	0.2266	0.3929

51.07 mg $Ni(CH_3COO)_2$ $V_\infty = 2463$ ml $K = 1.67 \cdot 10^{-3}$

Table 8. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using nickel acetate as a catalyst.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	80	0.0109	0.6558	0.3224	0.3084
60	173	0.0234	0.6434	0.3099	0.3173
90	268	0.0363	0.6304	0.2970	0.3268
120	355	0.0481	0.6186	0.2852	0.3362
150	439	0.0595	0.6072	0.2738	0.3459
180	520	0.0705	0.5962	0.2628	0.3558
210	594	0.0805	0.5862	0.2528	0.3653
240	664	0.0900	0.5767	0.2433	0.3749
270	732	0.0992	0.5675	0.2341	0.3846
300	794	0.1076	0.5591	0.2257	0.3940
330	855	0.1159	0.5508	0.2174	0.4037
360	910	0.1236	0.5431	0.2097	0.4133

83.01 mg $Ni(CH_3COO)_2$ $V_\infty = 2459$ ml $K = 2.21 \cdot 10^{-3}$

Table 9. The velocity constant for various amounts of nickel acetate.

mg	m.mol.	$\sqrt{\text{m.mol.}}$	$K \cdot 10^3$	$K^* \cdot 10^3$
31.76	0.1797	0.4239	1.28	3.02
51.07	0.2889	0.5375	1.67	3.11
83.01	0.4696	0.6853	2.21	3.22

Table 10. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using strontium acetate as a catalyst.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	26	0.0034	0.6633	0.3299	0.3033
60	66	0.0085	0.6582	0.3248	0.3068
90	105	0.0136	0.6531	0.3197	0.3103
120	142	0.0184	0.6483	0.3149	0.3136
150	183	0.0237	0.6430	0.3096	0.3174
180	222	0.0287	0.6380	0.3046	0.3211
210	260	0.0336	0.6331	0.2997	0.3248
240	296	0.0383	0.6284	0.2950	0.3284
270	340	0.0440	0.6227	0.2893	0.3329
300	369	0.0478	0.6189	0.2855	0.3360
330	401	0.0519	0.6148	0.2814	0.3394
360	438	0.0567	0.6100	0.2766	0.3434
390	470	0.0608	0.6059	0.2725	0.3470
420	504	0.0652	0.6015	0.2681	0.3509
450	533	0.0690	0.5977	0.2643	0.3544
480	560	0.0725	0.5942	0.2608	0.3576

86.2 mg $Sr(CH_3COO)_2$ $V_\infty = 2576$ ml $K = 0.85 \cdot 10^{-3}$

Table 11. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using strontium acetate as a catalyst.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	46	0.0060	0.6607	0.3273	0.3050
60	94	0.0122	0.6545	0.3211	0.3093
90	142	0.0185	0.6482	0.3148	0.3137
120	192	0.0251	0.6416	0.3082	0.3185
150	240	0.0313	0.6354	0.3020	0.3231
180	291	0.0379	0.6288	0.2954	0.3281
210	336	0.0438	0.6229	0.2895	0.3327
240	385	0.0503	0.6164	0.2830	0.3381
270	427	0.0557	0.6110	0.2776	0.3426
300	468	0.0610	0.6057	0.2723	0.3472
330	510	0.0665	0.6002	0.2668	0.3521
360	556	0.0725	0.5942	0.2608	0.3576
390	595	0.0776	0.5891	0.2557	0.3625
420	636	0.0830	0.5837	0.2503	0.3677
450	678	0.0885	0.5782	0.2448	0.3733
480	716	0.0934	0.5733	0.2399	0.3784

124.7 mg $Sr(CH_3COO)_2$ $V_\infty = 2556$ ml $K = 1.13 \cdot 10^{-3}$

Table 12. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using strontium acetate as a catalyst.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	52	0.0068	0.6599	0.3265	0.3056
60	114	0.0148	0.6519	0.3185	0.3111
90	172	0.0224	0.6443	0.3109	0.3165
120	237	0.0308	0.6359	0.3025	0.3227
150	295	0.0384	0.6283	0.2949	0.3285
180	357	0.0465	0.6202	0.2868	0.3349
210	412	0.0536	0.6131	0.2797	0.3408
240	466	0.0607	0.6060	0.2726	0.3470
270	520	0.0677	0.5990	0.2656	0.3532
300	571	0.0743	0.5924	0.2590	0.3593
330	620	0.0807	0.5860	0.2526	0.3655
360	665	0.0865	0.5802	0.2468	0.3712

206.6 mg $Sr(CH_3COO)_2$ $V_\infty = 2562$ ml $K = 1.39 \cdot 10^{-3}$

Table 13. The velocity constant for various amounts of strontium acetate.

mg	m.mol.	$\sqrt{\text{m.mol.}}$	$K \cdot 10^3$	$K^* \cdot 10^3$
86.2	0.419	0.647	0.85	1.31
124.7	0.606	0.779	1.13	1.45
206.6	1.004	1.002	1.39	1.39

Table 14. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using barium carbonate as a catalyst.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	32	0.0041	0.6626	0.3292	0.3038
60	78	0.0100	0.6567	0.3233	0.3078
90	133	0.0171	0.6496	0.3162	0.3127
120	190	0.0244	0.6423	0.3089	0.3179
150	242	0.0310	0.6357	0.3023	0.3229
180	296	0.0379	0.6288	0.2954	0.3281
210	350	0.0448	0.6219	0.2885	0.3335
240	398	0.0510	0.6157	0.2823	0.3387
270	446	0.0572	0.6095	0.2761	0.3439
300	496	0.0637	0.6030	0.2696	0.3496
330	542	0.0696	0.5971	0.2637	0.3550
360	596	0.0765	0.5902	0.2568	0.3614
390	642	0.0824	0.5843	0.2509	0.3671
420	689	0.0885	0.5782	0.2448	0.3733
450	729	0.0936	0.5731	0.2397	0.3785

350.1 mg $BaCO_3$ $V_\infty = 2597$ ml $K = 1.22 \cdot 10^{-3}$

Table 15. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using barium carbonate as a catalyst.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	47	0.0064	0.6603	0.3269	0.3052
60	107	0.0147	0.6520	0.3186	0.3110
90	165	0.0226	0.6441	0.3107	0.3167
120	225	0.0308	0.6359	0.3025	0.3227
150	282	0.0396	0.6271	0.2937	0.3294
180	340	0.0479	0.6188	0.2854	0.3361
210	396	0.0543	0.6124	0.2790	0.3414
240	449	0.0615	0.6052	0.2718	0.3476
270	499	0.0684	0.5983	0.2649	0.3538
300	552	0.0756	0.5912	0.2577	0.3606
330	603	0.0826	0.5841	0.2507	0.3673
360	650	0.0890	0.5777	0.2443	0.3738
390	700	0.0959	0.5708	0.2364	0.3810
420	745	0.1021	0.5646	0.2312	0.3877
450	790	0.1082	0.5585	0.2251	0.3946
	504.2 mg $BaCO_3$		$V_\infty = 2433$ ml	$K = 1.45 \cdot 10^{-3}$	

Table 16. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using barium carbonate as a catalyst.

min.	ml CO_2	x	$a-x$	$b-x$	$\log \frac{a-x}{b-x}$
0	0		0.6667	0.3333	0.3010
30	56	0.0074	0.6593	0.3259	0.3060
60	126	0.0166	0.6501	0.3167	0.3123
90	198	0.0260	0.6407	0.3073	0.3190
120	270	0.0355	0.6312	0.2978	0.3263
150	340	0.0447	0.6220	0.2886	0.3335
180	410	0.0539	0.6128	0.2794	0.3411
210	478	0.0629	0.6038	0.2704	0.3489
240	538	0.0707	0.5960	0.2626	0.3560
270	600	0.0788	0.5879	0.2545	0.3636
300	660	0.0867	0.5800	0.2466	0.3714
330	720	0.0946	0.5721	0.2387	0.3796
360	772	0.1014	0.5653	0.2319	0.3870
	693.9 mg $BaCO_3$		$V_\infty = 2536$ ml	$K = 1.72 \cdot 10^{-3}$	

Table 17. The velocity constant for various amounts of barium carbonate.

mg	m.mol.	$\sqrt{\text{m.mol.}}$	$K \cdot 10^3$	$K^* \cdot 10^4$
350.1	1.775	1.332	1.22	9.18
504.2	2.556	1.599	1.45	9.07
693.9	3.517	1.875	1.72	9.15

Table 18. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using potassium carbonate as a catalyst.

min.	ml CO_2	x	$b-x$	log $(b-x)$
0	0		0.3333	$\bar{1}.5228$
60	36	0.0048	0.3285	$\bar{1}.5165$
120	66	0.0087	0.3246	$\bar{1}.5114$
180	102	0.0135	0.3198	$\bar{1}.5049$
240	133	0.0176	0.3157	$\bar{1}.4993$
300	166	0.0220	0.3113	$\bar{1}.4932$
360	198	0.0262	0.3071	$\bar{1}.4873$
420	233	0.0309	0.3024	$\bar{1}.4806$
480	264	0.0350	0.2983	$\bar{1}.4747$

405.1 mg K_2CO_3 $V_\infty = 2515$ ml $K = 2.3 \cdot 10^{-4}$

Table 19. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using potassium carbonate as a catalyst.

min.	ml CO_2	x	$b-x$	log $(b-x)$
0	0		0.3333	$\bar{1}.5228$
30	56	0.0074	0.3259	$\bar{1}.5131$
60	80	0.0106	0.3227	$\bar{1}.5088$
90	110	0.0146	0.3187	$\bar{1}.5034$
120	141	0.0187	0.3146	$\bar{1}.4978$
150	174	0.0231	0.3102	$\bar{1}.4916$
180	207	0.0275	0.3058	$\bar{1}.4854$
210	238	0.0316	0.3017	$\bar{1}.4796$
240	272	0.0361	0.2972	$\bar{1}.4731$
270	300	0.0398	0.2935	$\bar{1}.4676$
300	333	0.0443	0.2890	$\bar{1}.4609$
330	366	0.0486	0.2847	$\bar{1}.4544$
360	401	0.0533	0.2800	$\bar{1}.4472$
390	435	0.0578	0.2755	$\bar{1}.4401$
420	472	0.0627	0.2706	$\bar{1}.4323$

1009.6 mg K_2CO_3 $V_\infty = 2510$ ml $K = 4.9 \cdot 10^{-4}$

Table 20. The reaction velocity for various amounts of potassium carbonate.

mg	m.equ.	$(\text{m.equ.})^{3/4}$	$K \cdot 10^4$	$K^* \cdot 10^4$
405.1	5.86	3.77	2.3	0.61
1009,6	14.61	7.48	4.9	0.65

Table 21. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using rubidium carbonate as a catalyst.

min.	ml CO_2	x	$b-x$	$\log (b-x)$
0	0		0.3333	$\bar{1}.5228$
60	48	0.0062	0.3271	$\bar{1}.5147$
120	72	0.0094	0.3239	$\bar{1}.5104$
180	102	0.0132	0.3201	$\bar{1}.5053$
240	132	0.0171	0.3162	$\bar{1}.5000$
300	160	0.0208	0.3125	$\bar{1}.4949$
360	191	0.0248	0.3085	$\bar{1}.4893$
420	218	0.0283	0.3050	$\bar{1}.4843$
480	248	0.0322	0.3011	$\bar{1}.4787$

750.4 mg Rb_2CO_3 $V_\infty = 2567$ ml $K = 2.0 \cdot 10^{-4}$

Table 22. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using rubidium carbonate as a catalyst.

min.	ml CO_2	x	$b-x$	$\log (b-x)$
0	0		0.3333	$\bar{1}.5228$
60	53	0.0070	0.3263	$\bar{1}.5163$
120	84	0.0112	0.3221	$\bar{1}.5080$
180	111	0.0147	0.3186	$\bar{1}.5033$
240	142	0.0189	0.3144	$\bar{1}.4975$
300	174	0.0231	0.3102	$\bar{1}.4916$
360	200	0.0278	0.3055	$\bar{1}.4850$
420	244	0.0324	0.3009	$\bar{1}.4784$

1005.9 mg Rb_2CO_3 $V_\infty = 2508$ ml $K = 2.4 \cdot 10^{-4}$

Table 23. The reaction velocity for various amounts of rubidium carbonate.

mg	m.equ.	$(\text{m.equ.})^{3/4}$	$K \cdot 10^4$	$K^* \cdot 10^4$
750.4	6.50	4.07	2.0	0.49
1005.9	8.71	5.07	2.4	0.47

Table 24. The reaction of ethyl diacetoacetate (0.1 mole) with glacial acetic acid (0.2 moles) at 120° using tetramethylammonium acetate as a catalyst.

min.	ml CO_2	x	$b-x$	log $(b-x)$
0	0		0.3333	$\bar{1}.5228$
60	24	0.0032	0.3301	$\bar{1}.5187$
120	48	0.0063	0.3270	$\bar{1}.5146$
180	70	0.0092	0.3241	$\bar{1}.5107$
240	99	0.0131	0.3202	$\bar{1}.5054$
300	125	0.0165	0.3168	$\bar{1}.5008$
360	147	0.0194	0.3139	$\bar{1}.4968$
420	172	0.0227	0.3106	$\bar{1}.4922$
480	197	0.0260	0.3073	$\bar{1}.4876$
1738.9 mg $(CH_3)_4N\ CH_3COO$		$V_\infty = 2528$ ml		$K = 1.7 \cdot 10^{-4}$

Table 25. The reaction velocity for different cations.

Cation	$K^*\ 10^3$	log K^*	r	$1/r$
Ba^{2+}	0.913	$\bar{4}.960$	1.35	0.74
Sr^{2+}	1.38	$\bar{3}.140$	1.13	0.92
Mn^{2+}	2.61	$\bar{3}.417$	0.80	1.25
Zn^{2+}	0.88	$\bar{4}.945$	0.74	1.35
Co^{2+}	3.50	$\bar{3}.544$	0.72	1.39
Ni^{2+}	5.12	$\bar{3}.494$	0.70	1.42
Mg^{2+}	5.31	$\bar{3}.725$	0.65	1.54
Li^{+}	0.258	$\bar{4}.412$	0.60	1.67
Na^{+}	0.159	$\bar{4}.201$	0.95	1.05
K^{+}	0.063	$\bar{5}.80$	1.33	0.75
Rb^{+}	0.048	$\bar{5}.68$	1.48	0.68

The author is indebted to Messrs. Pharmacia Ltd. for permission to publish this paper and to Fil. Mag. SOLVEIG HANSEN for assistance in the experimental work.

Research Laboratory of Pharmacia Ltd., Uppsala, November 1953.

REFERENCES

1. BRÄNDSTRÖM, A., Arkiv f. Kemi *6*, 201 (1953).
2. —— in press.
3. —— in press.
4. —— in press.
5. MARTELL, A. E., and CALVIN, M., Chemistry of the metal chelate compounds. Prentice-Hall, Inc. New York 1952, p. 207.

Tryckt den 6 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

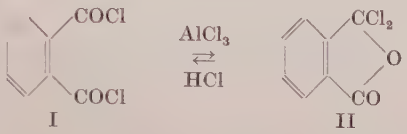
ARKIV FÖR KEMI Band 7 nr 15

Communicated 2 December 1953 by ARNE FREDGA

The reaction between phenylsuccinyl chloride and ammonia

By SIGVARD WIDEQVIST

It is a well-known fact that phthalyl chloride (I) is converted into the isomeric dichlorophthalide (II) in the presence of aluminum chloride:

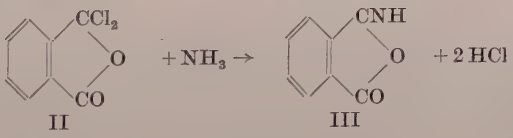


(1)

The dichlorophthalide is also called "unsymmetrical phthalyl chloride". In the absence of aluminum chloride, the dichlorophthalide is rearranged to phthalyl chloride. Hydrogen chloride is said to have a catalytic influence on this reaction (HÜCKEL (1)).

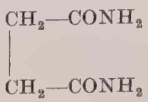
Succinyl chloride is also supposed to be similarly tautomeric, although this compound has not been obtained in the two postulated forms. The opinion that succinyl chloride is tautomeric is originally due to AUGER (2), who found that succinyl chloride on distillation at atmospheric pressure had no constant boiling point. It was, however, later established by VORLÄNDER (3), that the inconstancy of the boiling point depended on thermal decomposition; a tarry distillation residue was obtained. When the distillation was performed under reduced pressure, the boiling point was perfectly constant, and no distillation residue was obtained.

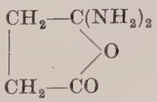
It has, however, been proposed that both succinyl chloride and phthalyl chloride have asymmetrical forms because of some of their reactions, *e.g.* with ammonia. When phthalyl chloride is treated with ammonia, the ammonium salt of o-cyanobenzoic acid is obtained, as found by HOOGEWERFF and VAN DORP (4). This reaction is assumed to take place with iminophthalide (III) as intermediate product. The formation of iminophthalide would be easily explained, if phthalyl chloride is assumed to react as dichlorophthalide:



(2)

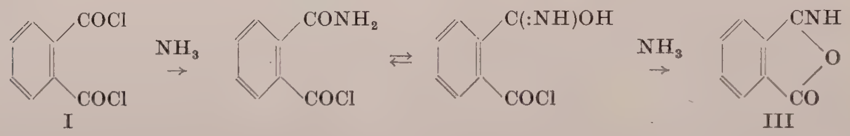
When succinyl chloride is treated with ammonia, two products are formed, namely succinamide (IV) and a substance which AUGER (2) assumed to be unsymmetrical succinamide (V).


IV


V

The unsymmetrical substance was the major product. On account of these observations AUGER concluded, that succinyl chloride contained only 9 per cent of the normal, symmetrical form. The "isoamide" obtained had curious properties. In contrast with succinamide it was very hygroscopic and easily soluble in water. When the aqueous solution was treated with silver nitrate, a silver salt was obtained having the formula $C_4H_4O_2NAg$. On acidifying the solution of the "isoamide", succinic acid was obtained.

However, neither the reaction of phthalyl chloride nor that of succinyl chloride with ammonia permits the assumption that the chlorides are unsymmetrical. The symmetrical chlorides might give the same products. Thus the iminophthalide (III) may be formed in the following way from the normal phthalyl chloride:

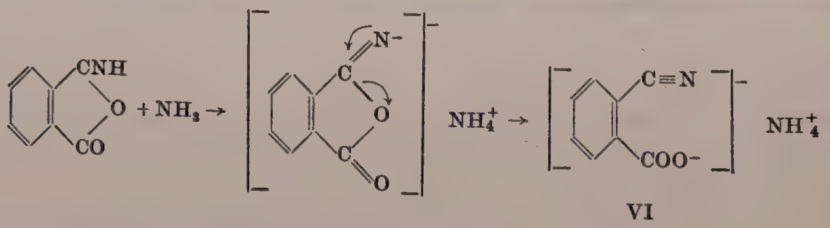


(3)

That this idea is correct seems obvious from the observations of SCHEIBER (5), who found that both phthalyl chloride and dichlorophthalide, on treatment with ammonia, gives o-cyanobenzoic acid. Phthalyl chloride, however, reacts several times as fast as dichlorophthalide. This probably implies, that the dichlorophthalide must be rearranged into phthalyl chloride, before it reacts with the ammonia.

It has been suggested by MORRELL (6) and VORLÄNDER (7) that the formation of the "isoamide" from succinyl chloride and ammonia takes place in a manner similar to reaction (3).

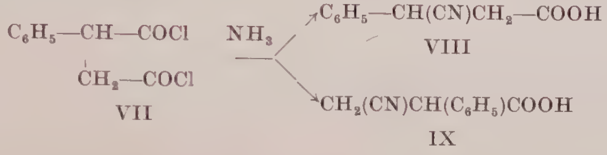
The formation of o-cyanobenzoic acid (VI) by the action of ammonia on phthalyl chloride is easily explained from an electronic theoretical point of view. The first product is iminophthalide, which under the influence of ammonia reacts as follows:



(4)

The product obtained by AUGER (2) on treatment of succinyl chloride with ammonia cannot be an amide. This was already pointed out by VORLÄNDER (7). It is far more likely, that the product is the ammonium salt of β-cyanopropionic acid. That succinic acid is obtained on acidifying the ammonium salt solution, is quite natural, as β-cyanopropionic acid is very easily hydrolyzed to succinic acid, as found by the present author (8). In order to detect the cyano acid, it must be very rapidly removed from the acidified solution. This is, however, somewhat difficult, as the β-cyanopropionic acid is very easily soluble in water. It can be mentioned, that Ives and Sames (9) prepared β-cyanopropionic acid by suspending its dry potassium salt in dry benzene; the salt was then decomposed by saturation with dry hydrogen chloride.

In order to get a more convenient reaction product it was preferred in the present investigation to study the reaction between ammonia and phenylsuccinyl chloride (VII). When the reaction takes place in the same way as in the case of phthalyl chloride, the reaction product will be phenylcyanopropionic acid. Because of the asymmetry of the phenylsuccinyl chloride molecule it would be two isomeric acids, namely β-phenyl-β-cyanopropionic acid (VIII) and α-phenyl-β-cyanopropionic acid (IX):



(5)

From the reaction mixture β-phenyl-β-cyanopropionic acid (VIII) was easily isolated. It is thus established, that succinyl chlorides also react with ammonia in the same manner as phthalyl chloride.

Experimental

A 250-ml three-necked flask, fitted with a mechanical stirrer, a thermometer, and a dropping funnel, was placed in an ice bath. Fourty milliliters of concentrated ammonia was placed in the flask and cooled to 0°. A mixture of 9 g of phenylsuccinyl chloride and 6 ml of dioxane was carefully added to the vigorously stirred ammonia solution. The temperature temporarily rose to 15°. When all the phenylsuccinyl chloride was added, the excess ammonia was evaporated *in vacuo*. The slightly yellowish solution was acidified with concd. hydrochloric acid. An oil separated and was extracted with ether. The ether was evaporated, and water was added to the residue, which was then treated with calcium carbonate under gentle warming. When all the oil had reacted with the carbonate, the solution was heated to boiling and rapidly filtered in order to remove excess calcium carbonate. From the filtrate a calcium salt separated on cooling. Yield 1.6 g. The salt was recrystallized from hot water with addition of some decolorizing charcoal. The calcium salt was decomposed with 2-N hydrochloric acid, and the liberated acid extracted with ether and dried with anhydrous sodium sulfate. When the ether was evaporated, a solid acid was obtained. The acid was recrystallized from benzene and identified as β-phenyl-β-cyanopropionic acid. M. P. 75–76°. A mixture of the reaction product with an authentic sample of β-phenyl-β-cyanopropionic acid showed the same melting point.

Anal. Subs., 33.31 mg: 9.95 ml of 0.01917-N $Ba(OH)_2$.
Calcd. for $C_{10}H_9O_2N$ equ. w., 175.2
Found equ. w., 174.7.

SUMMARY

The reaction between phenylsuccinyl chloride and ammonia has been studied. It was found, that the main reaction product was β-phenyl-β-cyanopropionic acid. On

account of this it is suggested, that the reaction product of succinyl chloride and ammonia, which was previously regarded as unsymmetrical succinamide, is the ammonium salt of β-cyanopropionic acid.

Laboratory of Organic Chemistry, University of Uppsala.

REFERENCES

1. Hückel, Theoretische Grundlagen der organischen Chemie I, Leipzig 1949, p. 197.
2. Auger, Bull. Soc. Chim. [2], *49*, 347 (1888).
3. Vorländer, Ann. *280*, 183 (1894).
4. Hoogewerff and van Dorp, Rec. trav. chim. *11*, 91 (1892).
5. Scheiber, Ber. *46*, 2366 (1913).
6. Morrell, J. Chem. Soc. *105*, 1734 (1914).
7. Vorländer and Kalkow, Ber. *30*, 2268 (1897).
8. Wideqvist, Arkiv Kemi. *3*, 289 (1951).
9. Ives and Sames, J. Chem. Soc. *1943*, 513.

Tryckt den 6 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 16

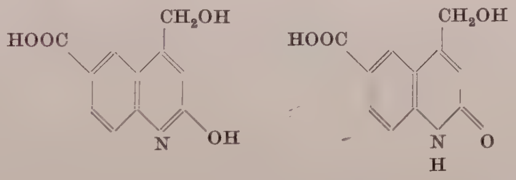
Mitgeteilt am 11. November 1953 durch H. v. Euler und K. Myrbäck

Zur Kenntnis der Dehydro-Ascorbinsäure

Von Hans Hasselquist

Mit 3 Figuren im Text

In einer vorhergehenden Mitteilung[1] wurde gezeigt, dass eine Dehydro-Ascorbinsäure-Lösung (DHA-Lös.), dargestellt durch Oxydation von Ascorbinsäure (Asc) mit p-Chinon, nach Zusatz von Anilin ein sehr unbeständiges farbloses Anil-Anilinsalz vom Schmpt 91–92° lieferte, das unter CO_2-Entwicklung leicht zu einer dunkelbraunen, schmierigen Masse zerfiel, die nicht zur Krystallisation gebracht und nicht identifiziert werden konnte. Wie Euler und Hasselquist[2] vorher nachwiesen, spaltet DHA beim Erhitzen in wässriger Lösung unter Bildung von Diketo-L-Gulonsäure (DKG) angenähert 1 Mol CO_2 ab. In der vorliegenden Arbeit wurde die Umsetzung mit p-Aminobenzoesäure an der entsprechenden DHA-Lösung bei 90° durchgeführt, wobei eine CO_2-Entwicklung erhalten wurde und gleichzeitig ein Reaktionsprodukt I auskrystallisierte, dessen Zusammensetzung mit derjenigen der 2-Oxy-4-oxymethyl-chinolin-carbonsäure-6 übereinstimmte.



I

Durch vorsichtige Oxydation von I mit CrO_3 in Eisessig wurde eine Oxydation der Oxymethylgruppe, die unter der Reaktion CO_2 abspaltete und in eine Substanz $C_9H_9O_6N$, übergeht. Durch Umsetzung von I mit Diazomethan wurde der entsprechende Methylester-methylether erhalten.

Von Euler und Hasselquist[3] sowie Hirst[4] liegen eingehende Studien vor über die Aufspaltung des in DHA bestehenden Laktonringes, die bei dessen Behandlung mit Alkali zu DKG oder durch langes Stehen der DHA-Lösung bei Zimmertemperatur eintritt. Hirst und Mitarb. zeigten dabei, dass eine solche Lösung unter Mutarotation[1] ein Gleichgewicht erreicht, in welchem die Lösung zu 85 % aus freier Säure, DKG, und zu 15 % aus Lakton, DHA, besteht.

---

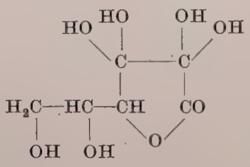
[1] H. Hasselquist, Dieses Arkiv, Bd. 4 Nr. 24, S. 369 (1952).

[2] H. v. Euler u. H. Hasselquist, Dieses Arkiv, Bd. 4 Nr. 38, S. 482 (1952).

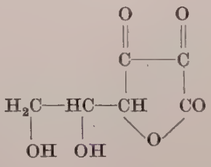
[3] H. v. Euler und H. Hasselquist, Dieses Arkiv, Bd. 5 Nr. 5, S. 49 (1952).

[4] E. L. Hirst und Mitarbeiter, J. Chem. Soc. 1933, 1270.

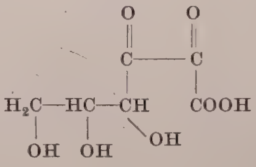
Bei der Oxydation von Asc zu DHA verwendete ich Jod oder, um eine injektionsreine Lösung zu erhalten, p-Chinon[2] als Oxydationsmittel. Um in den folgenden Versuchen eine DHA-Lösung spektrographisch näher zu studieren, wurde eine Methode nach Schauenstein[3] benutzt, nämlich Oxydation von Asc mit Wasserstoffperoxyd und Platin als Katalysator, da dieses Oxydationsmittel keine störende Eigenabsorption im Ultraviolett (UV) aufweist. Asc gibt im UV ein Maximum bei 245 mμ, das nach Oxydation, ausgeführt wie oben, schnell abnimmt und beim Übergang in DHA nach ca. 30 Min. ganz verschwindet (diese Lösung wird im folgenden mit A bezeichnet). Die Lösung verbraucht Dichlorphenolindophenol, Tillmans-Reagenz (TR) in saurer oder neutraler Lösung nicht. Diese Lösung dürfte zum grössten Teil aus DHA bestehen, aber nicht mit freien Keto-Gruppen, da diese im UV absorbieren würden; vielmehr schreibt Schauenstein dem DHA in wässriger Lösung die hydratisierte Form II zu.



II



III



IV

Nach 42-stündigem Verweilen der Lösung A bei Zimmertemperatur bildet sich allmählich ein neues Maximum bei 296 mμ aus, welches seinen höchsten Wert nach 17 Tagen erreicht, nämlich log $\varepsilon_{\max} = 2{,}58$. Schauenstein betont, dass dieses Endprodukt entweder als das wirkliche DHA und zwar entweder frei von Hydrat III oder frei von Lakton, DKG IV, anzusehen ist, welches das gleiche Maximum[4] geben dürfte.

Bei der Ausführung spektrographischer Versuche nach Schauenstein fanden wir gute Übereinstimmung zwischen unserem Maximum und Schauensteins Beobachtungen. (Fig. 1.)

Das DKG-Ba-Salz wurde nach Penny und Zilva[5] dargestellt; es wurde mit HCl angesäuert und das Spektrum wurde aufgenommen; dasselbe zeigte kein Maximum in UV.

Penny und Zilva[6] zeigten, dass Asc, DHA und DKG das identisch gleiche 2,4-Dinitro-phenylosazon gaben, was auch durch die Umsetzung mit Phenylhydrazin[7] bestätigt wurde. Wurde die nach 17 Tagen umgelagerte DHA-Lösung, deren Maximum bei 296 mμ verblieb, die nach SCHAUENSTEIN III oder IV enthalten sollte, mit Phenylhydrazin umgesetzt, so ergab sich nicht das entsprechende Osazon für Asc, DHA oder DKG, sondern das Pyrazolon V, welches OHLE und BÖCKMANN[1] durch Behandlung von Asc-Osazon mit Alkali erhalten hatten.

---

[1] Vgl. F. Petuely, Über Mutarotation. Angew, Chemie 65, 268 (1953).

[2] J. W. Pattersen, J. Biol. Chem. 183, 81 (1950).

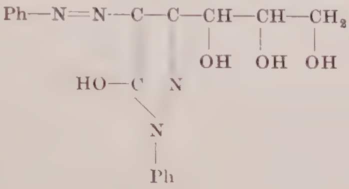
[3] E. Schauenstein, Mh. Chem. 79, 487 (1948).

[4] Vgl. E. Schauenstein, Mh. Chem. 80, 83 (1949) sowie die Absorption konjugierter CO-Chromophore, J. Bielecky u. V. Henry, Ber. 47, 1712 (1914).

[5] J. R. Penny u. S. S. Zilva, Biochem. J. 39, 2 (1945).

[6] J. R. Penny u. S. S. Zilva, Biochem. J. 37, 404 (1943).

[7] H. Hasselquist, Dieses Arkiv, Bd. 4 Nr. 24, S. 375 (1952).



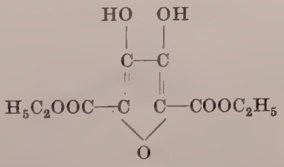
V

Wurde die Lösung B 15 Minuten auf dem Wasserbad erhitzt und danach aufs neue spektrometriert, so stieg bei 296 mμ log $\varepsilon_{\max}$ von 2,38 auf 3,68 (Lösung C), und gleichzeitig bildete sich auch ein Maximum bei 238 mμ aus, welches als Asc durch Papierchromatographie identifiziert werden konnte sowie durch die Fähigkeit der Lösung Tillmans-Reagenz (TR) in neutraler oder saurer Lösung zu entferben. (Fig. 2.)

Wurde die Lösung A auf dem Wasserbad in Gegenwart von HCl 10 Min. erhitzt, so entstand eine CO_2-Entwicklung.

Grössere Mengen Ascorbinsäure konnten spektrometrisch nachgewiesen werden; dagegen entstand sehr wenig von der Substanz mit dem Maximum 296 mμ. In der Lösung A, in welcher DHA-Hydrat und vermutlich DKG-Hydrat vorkommen, wird eine Rückhydrierung von DHA und DKG zu Asc in Gegenwart von HCl beobachtet. Der erforderliche Wasserstoff wird wahrscheinlich vom DKG geliefert, welches vermutlich enolysiert[2] ist und ein Endiol bildet, dessen Wasserstoff in DHA übergeführt wird.

Die in der Lösung B befindliche Substanz ist möglicherweise ein Dihydro-furan-Derivat, das bei der Erhitzung der Lösung C unter Wasserabspaltung in ein Furan-Derivat übergeht. Spektrographisch wurde die Kurve des von JOHNSSON u. JOHNS[3] dargestellten 3,4-Dioxy-furan-2,5-dicarbonsäureethylester VI aufgenommen.



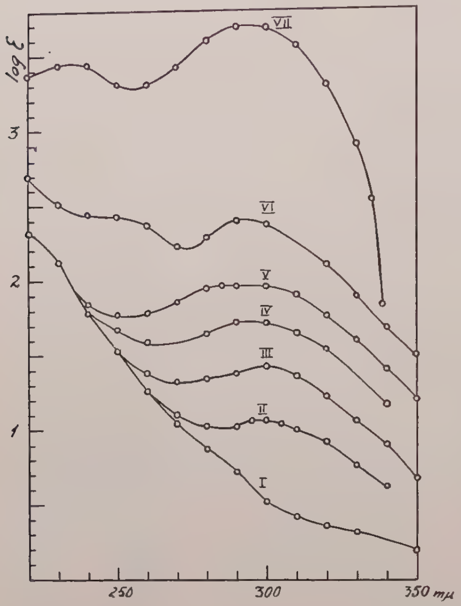
Dieses zeigte ein Maximum im UV bei 284 mμ und log $\varepsilon = 4{,}25$. Beim Eindunsten der Lösung C im Vacuum wurde ein beinahe farbloses schmieriges Öl erhalten, das nicht zur Krystallisation gebracht werden konnte. Versuche sind im Gang, aus demselben ein definierbares Derivat zu gewinnen. Es zeigte sich, dass die Substans in Lösung C ($E_{\max} = 296$ mμ) mit Wasserdampf leichtflüchtig war.

---

[1] H. OHLE u. G. BÖCKMANN, Ber. 67, 1751 (1934).

[2] H. v. EULER, Dieses Arkiv, Bd. 4, Nr. 7, S. 172 (1952).

[3] T. B. JOHNSSON u. JOHNS, J. Am. Chem. Soc. 36, 293 (1936).



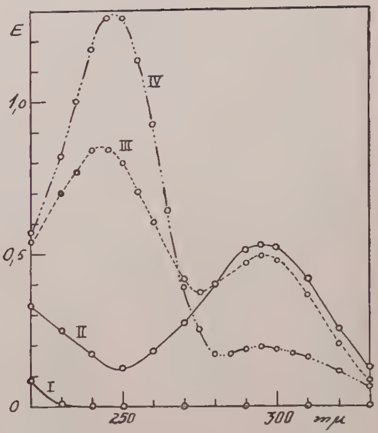


Fig. 3. UV-Spectra der chromatographierten Lösungen der Fig. 2.

Fig. 1. UV-Spektrum von 1 Mol Ascorbinsäure + 2 Mol H_2O_2 + Pt nach verschiedenen Zeiten bei 20°. Die Lösung ist 0,01-mol. bezgl. Asc.

Molare Extinktion $\varepsilon = \frac{E}{c.\ 1{,}0\ \text{cm.}}$

Kurve I 18 Stunden (DKG-Ba-Salz, dargestellt nach PENNY u. ZILVA, versetzt mit HCl, gab das gleiche UV-Spektrum wie I).
Kurve II 2 Tage.
Kurve III 3 Tage.
Kurve IV 4 Tage.
Kurve V 7 Tage.
Kurve VI 17 Tage und länger.
Kurve VII Lös. VI auf dem siedenden Wasserbad 15 Min. erhitzt.

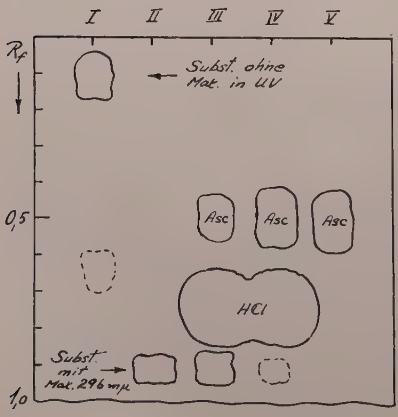


Fig. 2. Papierchromatogramm der 0,01-m Dehydro-Ascorbinsäure, annähernd identisch mit Lösung I in Fig. 1.

I. Ursprüngliche Lösung nach 1 Tag.
II. Lösung I, erhitzt auf 100° während 10 Min.
III. Lösung II, versetzt mit HCl (bis gesamt 8 %) und wieder 10 Min. auf 100° erhitzt.
IV. Lösung I, versetzt mit HCl (bis gesamt 8 %) und 10 Min. auf 100° erhitzt.
V. 0,01-mol. Ascorbinsäure.

Chromatographierungs-Lös.: 40 ml Butanol + 10 ml Eisessig + 10 ml Wasser. Das Chromatogramm wurde hervorgerufen durch Bespritzen des Papiers mit TR-Lösung, in 96 %igem Alkohol; dabei färbte sich das Papier schwach rot. Durch nachfolgende Behandlung des Papiers mit NH_3-Dampf traten die Flecken weiss auf blauem Grund auf.

Beschreibung der Versuche

Umsetzung der Dehydro-Ascorbinsäure mit p-Aminobenzoesäure zu 2-Oxy-4-oxymethyl-chinolincarbonsäure-6 (H.H. 199)

1,76 g Ascorbinsäure, in 5 ml Wasser gelöst, wurden mit einer Ätherlösung geschüttelt, welche 1,05 g p-Chinon enthielt, 1,4 g p-Aminobenzoesäure in 5 ml Wasser + 0,5 ml konz. HCl gelöst wurde mit der Dehydro-Ascorbinsäure-Lösung versetzt. Die Mischung wurde auf dem Wasserbad solange erwärmt, als die CO_2-Entwicklung anhielt. Eine Substanz fiel während der CO_2-Entwicklung aus. Diese Fällung wurde mit Wasser gewaschen, mit etwas Methanol verrieben, erwärmt, dann abgekühlt und abfiltriert. Ausbeute: 0,5 g, Schmpt: 270°. Die Substanz ist leicht löslich in Dioxan, löslich in warmen Methanol, Ethanol und Aceton.

Analysen: H.H. 199	Für $C_{11}H_9O_4N$				
		ber.	C = 60,3 %	H = 4,11 %	N = 6,40 %
		gef.	60,8	4,31	5,72

Methylierung von H.H. 199 mit Diazomethan (H.H. 200)

100 mg Rohprodukt wurden mit einem Überschuss von in Äther gelöstem Diazomethan versetzt, wobei sich eine lebhafte Stickstoffentwicklung zeigte. Nach 60 Minuten, als die Stickstoffentwicklung nur noch schwach war, wurde ein ungelöstes braunes Pulver abfiltriert, das Filtrat wurde im Vacuum bei 20° eingedunstet.

Das Reaktionsprodukt krystallisierte dabei in Form schwachbrauner spiralförmiger Nadeln aus, welche abfiltriert mit wenigen Tropfen Äthylacetat gewaschen wurden. Schmpt: 147–150° C. Ausb. 60 mg.

Umkrystallisiert aus wenig Äthylacetat, Schmpt: 130–140°; umkrystallisiert aus Äthanol, Schmpt: 155–156,5° ohne Zersetzung.

Schildförmige Krystallblätter, umkrystallisiert aus Methanol ergeben den Schmpt 156,5–157°.

Analysen: H.H. 200	Für $C_{13}H_{13}O_4N$				
		ber.	C = 63,2 %	H = 5,26 %	N = 5,67 %
		gef.	63,4	4,67	5,58

Oxydation von H.H. 199 mit CrO_3 (H.H. 201)

450 mg Substanz, aufgelöst in 8 ml Eisessig, wurden mit einem Überschuss von CrO_3 versetzt. Das Ganze wurde vorsichtig auf dem Wasserbad erwärmt, bis die Reaktion in Gang kam, und hierauf bei 20° stehen gelassen. Durch die Reaktionswärme wird die Lösung einige Minuten im Sieden gehalten. Nach Abkühlung wurde die Lösung mit 4 Volumina Wasser verdünnt und mit Äthylacetat ausgeschüttelt. Schmpt des Reaktionsproduktes 226° unter Zersetzung.

Leichtlöslich in Methanol, löslich in warmem Äthylacetat und in Aceton, schwerlöslich in Wasser.

Analysen: H.H. 201	Für $C_9H_9O_6N$				
		ber.	C = 47,58 %	H = 3,99 %	N = 6,17 %
		gef.	48,24	4,06	6,32

Umsetzung der Ascorbinsäure + H_2O_2 + Pt nach 17 Tagen (Lösung B) mit Phenylhydrazin

10 ml 0,01-m Lösung wurden mit (17,60 mg) Substanz, 200 mg Phenylhydrazin-HCl + 200 mg NaAc + 0,1 ml Eisessig versetzt und die Mischung bei 20° stehen gelassen.

Es fielen gelbrot gefärbte Nadeln aus, die mit Wasser gewaschen wurden. Nach zweimaligem Umkrystallisieren aus Aceton war der Schmelzpunkt 210–211°.

Ein Mischschmelzpunkt mit dem von OHLE und BÖCKMANN dargestellten Pyrazolon gab keine Depression. Das Spektrum im UV zeigte sich in beiden Substanzen identisch, Maximum bei 250 mμ.

ZUSAMMENFASSUNG

Bei der Umsetzung von Dehydro-Ascorbinsäure mit p-Aminobenzoesäure in wässriger Lösung entsteht unter CO_2-Entwicklung 2-Oxy-4-oxymethyl-chinolincarbonsäure.

Es wird gezeigt, dass Ascorbinsäure, Dehydro-Ascorbinsäure und Diketo-Gulonsäure mit oder ohne Hydrat mit Phenylhydrazin das gleiche Osazon geben, während die unter 17tägigem Stehen oxydierte Ascorbinsäure-Lösung kein Osazon sondern *nur* Pyrazolon V gab (Formel S. 123). Möglicherweise findet während dieser Zeit eine Umlagerung statt, ev. in ein furanähnliches Derivat unter Ringschluss zwischen den C-Atomen 2 und 5 oder 3 und 6. Die Carboxylgruppe, die hier frei zu sein scheint, gibt CO_2 beim Erhitzen auf 100° ab; das dabei entstehende Endprodukt vom Absorptions-Maximum 296 mμ ist beständig gegen Sieden in 8 %iger HCl (siehe Fig. 2) und zeigt keine Neigung mit Phenylhydrazin ein Osazon zu bilden. Ist mit Wasserdampf leichtflüchtig.

Die Lösung dieses Endproduktes enthält auch etwas Dehydro-Ascorbinsäure oder Diketo-Gulonsäure, da auch Ascorbinsäure zurückgebildet wird. Die molare Extinktion der Endsubstanz verbleibt dabei unverändert (siehe Fig. 3).

Herrn Ingenieur GUNNAR HANSHOFF danke ich für die Ausführung der spektrographischen Untersuchungen in dieser Arbeit.

Stockholm Universität, Institut für organisch-chemische Forschung.

Tryckt den 6 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 17

Communicated 24 February 1954 by John Runnström and Karl Myrbäck

Proteolytic activity in sea urchin gametes

IV. Further investigations of the proteolytic enzymes of the egg

By Gunnar Lundblad

With 3 figures in the text

In previous papers (1, 2, 3) the proteolytic activity of spermatoza and unfertilized eggs of different sea urchin species has been treated, as well as the appearance of the increased enzymatic activity of fertilized eggs a few minutes after fertilization. The proteolytic activity has in these experiments mainly been determined viscosimetrically, but formol titrations have also been carried out and in some cases the benzoylarginine amide splitting activity has been measured. Activity optima at three different pH-values have been found in extracts from unfertilized eggs. Cysteine activates the enzyme with an optimum at pH 4.3 (cathepsin II type) and at pH 6.7 (the enzyme is called EI), but has in certain extracts an inactivating effect at pH around 7 (the enzyme is called EII). Besides there is a protease with a pH optimum at 7.8 (cf. (3)).

It has earlier not been possible to decide whether the last-mentioned activity can be referred to the enzyme EII or not. An investigation to this effect has therefore been one of the subjects of the present study. Another subject of interest was to find out whether any other enzyme but EI (active at pH 6.7) is activated upon fertilization. Besides, a minor investigation has been carried out on a proteolytic enzyme with an optimum activity at about pH 8.5–9.0, the so-called hatching enzyme which appears shortly before the development of the egg reaches the blastula stage. This enzyme has previously been studied in other sea urchin species (4, 5, 6). The proteolytic activity appearing in some jelly coat solutions (1) has been explained and most likely identified and a scheme of the author's concept about the appearance of the proteolytic enzymes at fertilization is presented.

Experimental

Material and methods

Material from eggs of *Paracentrotus lividus* and *Arbacia lixula* from the Gulf of Naples and from the coast of Banyuls-sur-Mer has been used. The work on these species has been carried out at the Stazione Zoologica, Naples, and at

Laboratoire Arago of Banyuls during the spring and early summer 1953. Eggs from *Echinocardium cordatum* from the Swedish west coast were also investigated during the summer 1953 at Kristineberg's Zoological Station. The egg material was obtained by cutting the sea urchins in the ordinary way. As to *Paracentrotus* and *Echinocardium* the rinsed gonades were placed in filtrated sea water for spooning of the ripe eggs, whereas the entire open half of *Arbacia* with the rinsed ovaries was placed in a Boveri disc filled with sea water for spontaneous spooning. The egg material was washed 2–3 times in sea water and in some cases in isotonic sodium chloride solution as well. After suspension in a medium (about twice the volume of the egg material) it was homogenized by sucking some times through glass filter (G1 for *Arbacia* and *Echinocardium* and G2 for *Paracentrotus*). The egg material and homogenization fluid had been cooled to + 3° C and suction of air had been avoided. The cytolyse brought about by homogenization was checked under the microscope. Thereafter the material was centrifuged for approx. half an hour at + 3° C (*c.* 5 000 g). In some cases the eggs were frozen after washing, thawed and centrifuged. These centrifuged solutions have in the present paper been called *extracts,* to be distinguished from earlier papers where they were called *homogenates* also after centrifugation, which seems less correct.

Except for the use of lyophilized *Arbacia* eggs in heparin experiments, all investigations on eggs have in this work been carried out on fresh not lyophilized material. The jelly coat preparations studied have been partly lyophilized and partly fresh, not lyophilized.

The proteolytic activity has in this paper been viscosimetrically determined with gelatin as substrate. The procedure (cf. (1, 2, 3,)) is briefly as follows. The substrate consists of 4.00 per cent solutions of gelatin (Fischer Sc. Co.) containing 0.005 per cent merthiolate (Lilly) as a sterilizing agent, and buffered either in acetate (pH 4.3), imidazol (pH 6.7 and 7.2), or veronal (pH 7.8 and 8.5–9.0) in a molar concentration of 0.1. The activity was measured in Ostwald viscosimeters at 35.5° C, calculated according to a formula by HULTIN[1] (7) and expressed as $m\mu A$, where A is the activity unit ($m\mu A = A \times 10^{-9}$).

The samples which should be measured consisted of 1.00 ml extract and 3.00 ml gelatin solution, or of 1.00 ml extract, 0.50 of a reagent (water, cysteine solution or another solution) and 3.00 ml gelatin solution. The mixing of extract (in certain cases + reagent) and gelatin is called the start. In several cases the activity of 1.00 ml extract mixed with 3.00 ml gelatin is first measured and the activity is then called $m\mu A^{\mathrm{I}}$. After a certain time 0.50 ml of a *0.01 M cysteine* solution (pH always adjusted to 6.5) is added and the measurement continued. This new value of activity is called $m\mu A^{\mathrm{II}}$ (the enzyme dilution factor in HULTIN's formula has of course to be changed).

---

[1] HULTIN's formula: $A^{t^\circ} = J \cdot c^2{}_s \cdot \dfrac{d\dfrac{1}{\eta_{sp}}}{d\,t}$

where A = the enzymic acitivity at $t°$ C (units per gram) in the reaction mixture,
J = a factor for the influence of e.g. pH (for most substrates J = 1; independent of pH).
c_s = the concentration of the substrate in the reaction mixture (gram substrate per gram solution),
η_{sp} = specific viscosity and
t = time in seconds.

The pH is always checked by Beckman glass electrode pH meter, model G, and the nitrogen is determined according to Kjeldahl (a modified micro method), Nesslerization and measuring in Beckman spectrophotometer.

Results

I. Investigation of the proteolytic enzymes with activity at neutral pH

As is seen in Fig. 1, which gives the results of a previous work (3), the author has demonstrated a pH optimum at pH 6.7 and one at pH 7.8 by means of formol titration of a cysteine treated centrifuged homogenate of fresh unfertilized *Arbacia* eggs. When investigating the catheptic activity with benzoylarginine amide as a substrate in acid solution, it was shown in the same paper that a cysteine activated extract of lyophilized unfertilized *Paracentrotus* eggs (see Fig. 5 (3)) showed activity at pH 6.7 and around pH 8, apart from the catheptic activity at pH 4.3. These results seem to indicate that it is also possible to activate the two non-catheptic enzymes with cysteine and these are consequently of SH type. In another earlier paper ((2), Tables 4 and 5) the existence has been demonstrated of two enzymes in extracts from lyophilized *Arbacia* eggs, and with proteolytic activity at neutral pH. The enzymes were called EI and EII. The enzyme which was activated with cysteine and obtained through short extraction (measured at pH 6.6) was designated EI. The enzyme which was obtained after prolonged extraction including freezing (Tables 4, 5) was called EII. The activity of enzyme EII considerably decreased after addition of cysteine (the activity was measured at pH 6.9 and 7.2). Even in centrifuged solutions of homogenized fresh unfertilized *Arbacia* eggs a strong proteolytic activity (at pH 6.6) sometimes appeared but was almost entirely removed by cysteine (cf. Table 16, (2)). The thiol enzyme EI, the activity of

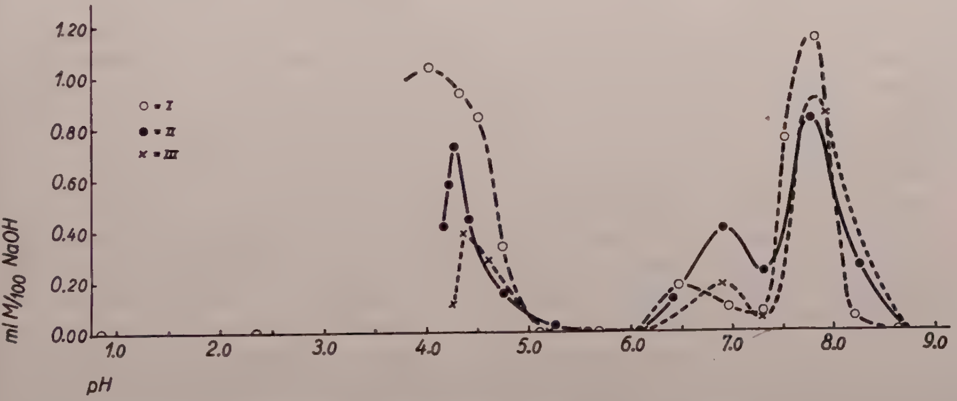


Fig. 1. Activity-pH-curves made on homogenates from fresh unfertilized *Arbacia* eggs. Formol titration. For details see (3).

I: Distilled water homogenate from unfertilized *Arbacia* eggs.
II: The same homogenate stored two days in the cold before measurement.
III: The same kind of homogenate made on another batch of eggs.

which has been observed at pH 6.6 must correspond to the proteolytic activity between pH 6 and 7 in Fig. 1 or at least part thereof. The hypothesis now presents itself that part of this activity also may be due to enzyme EII, the effect of which has also been demonstrated at pH 6.6 but whose optimal pH is so far unknown, due to inferences of other proteolytic enzymes and inhibitors which may be present. Although this enzyme is inhibited in crude extracts by cysteine it may be assumed that some of the activity remains in the presence of cysteine, if we postulate that the inhibition is caused by some inhibitor which is activated by cysteine.

A. The proteolytic activity at pH 6.7 (EI, EII) and at 7.8

The following experiments have been carried out on extracts from eggs washed in sea water or in isotonic sodium chloride solution. Thereafter the material was homogenized by suction of the egg suspension through glass filter. The results are recorded below and in Table 1.

Unfertilized *Paracentrotus* eggs were washed three times in sea water, homogenized in three times its sedimented volume of distilled water, and centrifuged. The samples were mixed with gelatin and measured. Two and a half hours later cysteine was added and the measurements were continued. At pH 6.7 as well as at pH 7.7 the activity of the cysteine samples decreased. This seems to indicate the presence of enzyme EII in the extracts. In order to eliminate the release of EII which may have taken place due to the presence of ions in the sea water, the following series of experiments was made with eggs washed in sodium chloride. The results are recorded in Table 1. Also here unfertilized eggs of *Paracentrotus* were used. These were not only washed in sea water, but also three times in isotonic sodium chloride solution. The equal volume of distilled water was added to the sedimented eggs which were thereafter homogenized and centrifuged. It is evident from Table 1 that the activity at pH 6.7 somewhat increases by cysteine but decreases to $^1/_3$ by iodine solution. At pH 7.6 the activity is higher, it is doubled at cysteine treatment and decreases 50 per cent due to the influence of iodine. The values indicate the appearance of EI at pH 6.7 and of another enzyme which can be activated at pH 7.8, and which will subsequently be called EIII. Due to the preparative washing of the egg material EII has apparently never been released.

Table 1. The enzymatic activity in extracts from *Paracentrotus* eggs washed in isotonic sodium chloride solution.

1.00 ml extract was incubated with 0.50 ml reagent at 20° C for 30 minutes (pH 6.5), then mixed with 3.00 ml gelatin and measured. $N_{extr} = 1.60$ mg/ml.

Expt. No.	Reagent	pH	mμA/ml	mμA/mg N	Rel. activity
1	H_2O	6.7	5.3	3.3	1.00
2	Cysteine	6.7	6.4	4.4	1.21
3	I_2–KI, 0.01 N	6.7	2.1	1.3	0.40
4	H_2O	7.6	20	12	1.00
5	Cysteine	7.6	40	25	2.00
6	I–KI, 0.01 N	7.6	8.5	5.3	0.43

Previous physiological studies have revealed that hypertonic treatment of the egg material brings about parthenogenesis. In an earlier paper ((2) Tables 21–23) it was found that such treatment has, after addition of cysteine, an activating effect on the enzyme active at pH 6.7. Experiments have been carried out simultaneously with the two homogenization media, distilled water and 1 M potassium chloride. In Table 4 (2) it was shown that extraction in 1 M KCl brings about 4–5 times higher activity at pH 6.4, compared to the activity in distilled water extract. This activity is evidently caused by the enzyme EII. The results of the experiments carried out in this study are recorded in Tables 2 and 3. Table 2 concerns *Paracentrotus* and Table 3 *Arbacia*.

Unfertilized *Paracentrotus* eggs were washed three times in sea water and one hour in a mixture of 60 ml 2.5 M KCl and 200 ml sea water. The sedimented egg material was devided in two parts, (A) Homogenization in distilled water, and (B) homogenization in the same volume 1 M KCl (MIRSKY). The homogenates were then centrifuged. The nitrogen content was in (A) 0.75, and in (B) 0.83 mg/ml.

Table 2. The proteolytic activity in extracts from *Paracentrotus* eggs treated with hypertonic sea water.

1.00 ml extract was mixed with 3.00 ml gelatin and measured (A^{I}). After 90 minutes 0.50 ml cysteine was added and the measurements continued (A^{II}).

Expt. No.	Homogenization medium	pH	mμA^{I}/ml	mμA^{I}/mg N	mμA^{II}/ml	mμA^{II}/mg N	$\frac{A^{II}}{A^{I}}$
7	H_2O	6.7	0	0	16	21	—
8	1 M KCl	6.7	0	0	20	24	—
9	H_2O	7.2	62	83	66	88	1.06
10	1 M KCl	7.2	48	58	18	22	0.37
11	H_2O	7.8	29	39	23	31	0.79
12	1 M KCl	7.8	104	125	19	23	0.18

Table 2 demonstrates that at pH 6.7 there is no original activity in the extracts. Some activity is, however, brought about after addition of cysteine. This indicates the occurrence of EI in both extracts. At pH 7.2 activity occurs without addition of cysteine and the activity is strongest in the distilled water extract. Cysteine addition somewhat increases the activity in the distilled water extract at pH 7.2, whereas the activity decreases in the KCl extract. This seems to indicate that EI predominates in the distilled water extract and EII in the KCl extract. At pH 7.8, the activity is low in untreated distilled water extract and does not increase after addition of cysteine. In the KCl extract the activity is very high in cysteine-free sample but quickly decreases after addition of cysteine. This should give further support to the assumption that EI mainly predominates in the distilled water extract and EII in the KCl extract.

Unfertilized eggs from *Arbacia* were washed three times in sea water. The sedimented egg material was then divided in two equal parts. (A) Homogenization in distilled water, and (B) homogenization in 1 M KCl. After homogenization the material was centrifuged. The nitrogen content was: (A) 1.08 mg/ml, (B) 1.80 mg/ml.

Table 3. The proteolytic activity in extracts from *Arbacia* eggs homogenized in distilled water or potassium chloride.

1.00 ml extract was mixed with 3.00 ml gelatin and measured (A^{I}). Two hours later 0.50 ml cysteine was added and the measurements continued (A^{II}). An asterisk indicates abnormally rapid sinking activity.

Expt. No.	Homogenization medium	pH	mμA^{I}/ml	mμA^{I}/mg N	mμA^{II}/ml	$\frac{\text{m}\mu A^{II}}{\text{mg N}}$
13	H_2O	6.7	0	0	0	0
14	1 M KCl	6.7	92*	51*	0	0
15	H_2O	7.2	14	13	0	0
16	1 M KCl	7.2	34	19	30	17
17	H_2O	7.7	29	27	0	0
18	1 M KCl	7.7	50*	28*	0	0

The results in Table 3 reveal that there is no spontaneous activity of EI in the distilled water extract at pH 6.7, nor after the addition of cysteine. In the KCl extract the activity at this pH is strong, yet it is instable and cysteine has no increasing effect. This may indicate the presence of EII. At pH 7.2 the activity in the distilled water extract is low and even here cysteine has no increasing effect. In the KCl extract the activity is higher and stable; it does not seem to be influenced by cysteine. At pH 7.7 the activity is doubled in the KCl extract, but in both extracts it soon disappears and cannot be reactivated with cysteine. The fact that cysteine does not activate may imply that EI was destroyed before addition of cysteine. At all presented pH values the activity is higher in the KCl extracts. Transient activity is often characteristic for the enzymes of the *Arbacia* extracts.

As reported earlier (p. 399 (3)) it was found that heparin (0.03–0.06 per cent solution, Hynson, Westcott, and Dunning Co., lot no. 218) had no effect upon the enzymes active at pH 4.3 and 6.7. The latter enzyme was found to be the thiolenzyme EI. Now it was considered of interest to know if there was any effect upon the enzyme EII (inactivated by cysteine) which has shown to be of importance at fertilization. As reported by HARDING and HARDING (8) addition of heparin a few seconds after sperm addition resulted in reduced fertilization (50 per cent) and addition at a later time (30 seconds or more) did not prevent fertilization, but no elevation of the fertilization membrane oc-

Table 4. Influence of heparin upon the enzyme EII in extract of *Arbacia* eggs.

1.00 ml extract was incubated with 0.50 ml reagent for 1 hour at 20° C and then mixed with 3.00 ml gelatin and measured.

Expt. No.	Reagent	pH	mμA/ml	mμA/mg N	Rel. activity
19	H_2O	6.8	9.3	11	1.00
20	Cysteine	6.8	1.5	1.8	0.16
21	Heparin, 0.05 %	6.8	2.0	2.4	0.22
22	H_2O	7.6	4.2	5.0	0.45

curred. Therefore the experiments with heparin (same preparation) were repeated when extracts were obtained in which only the enzyme EII was active. It was found that heparin had a strong inactivating effect upon EII. One of the experimental series is shown in Table 4.

The material was lyophilized *Arbacia* eggs stored 10 months at — 20° C and then extracted in H_2O, frozen, thawed, kept at + 3° over night, centrifuged and then dialysed for 24 hours against 0.9 per cent sodium chloride solution. N = 0.85 mg/ml.

B. A study of the enzyme active at pH 7.8 (EIII)

The enzymatic activity around pH 7.8 which was studied in Table 1 and defined as a special enzyme which could be activated with cysteine, was called EIII. It has been the subject of several investigations and the results are presented in Tables 5–12. Among the substances used to prove the presence of EIII, alloxan was of particular importance. These experiments are recorded in Tables 5 and 8–12. The reason why alloxan was used is that fresh solution of this substance was one of the few reagents which, as proved by the author, has a considerable activity-inhibiting effect on proteolytic enzymes from sea urchin spermatozoa (9). It then seemed of interest to investigate whether proteolytic enzymes from the egg were also influenced.

Unfertilized *Paracentrotus* eggs were washed three times in sea water and homogenized in this medium. The suspension was left for half an hour in the cold before centrifugation. N = 2.65 mg/ml. When measuring the proteolytic activity at pH 6.7 and 7.7 the values were 74 and 140 mμ*A*/ml. This extract was further studied and the results are recorded in Table 5. It is evident from Table 5 that no activation was caused by alloxan alone. When adding cysteine to alloxan, however, the activity was doubled, compared to the activity when adding cysteine alone or 2.5 times stronger than the original activity. It is possible that alloxan destroys an inhibitor which is active at pH 7.7 and blocks part of the enzyme EIII. At the same time addition of sulphydryl groups is, however, required in order to render the enzyme active which was "liberated" through the influence of alloxan. It is of interest to compare this with the effect on the enzyme EII which is not activated by SH groups. As shown in Tables 18 and 19, EII is activated by alloxan without addition of cysteine.

Table 5. The influence of alloxan upon the proteolytic activity of *Paracentrotus* egg extract.

1.00 ml extract was incubated with 0.20 ml of reagent I for 20 minutes at 20° C, whereupon 0.30 ml of reagent II was added and the incubation continued for 20 minutes at 20° before 3.00 ml gelatin was added and the measurement started. Alloxan 0.01 M (fresh solution). Cysteine 0.01 M. pH corrected to 6.5 in the reagents. pH at the measurements 7.7.

Expt. No.	Reagent I	Reagent II	mμ*A*/ml	mμ*A*/mg N
23	H_2O	H_2O	41	16
24	Alloxan	H_2O	41	16
25	Alloxan	Cysteine	105	40
26	H_2O	Cysteine	54	20

In previous experiments with extracts from unfertilized eggs of *Echinocardium cordatum* there was no activity at neutral pH (6.7). Later investigations of the proteolytic activity at pH 7.8 revealed that considerable activity may occur. The results of these investigations are presented in Tables 6–12.

Unfertilized eggs of *Echinocardium* were washed three times in sea water, homogenized in sea water + distilled water (1 : 1) and centrifuged. The samples were mixed with gelatin and measured. The activity at pH 6.7 was zero also when adding cysteine two hours after the start. The activity at pH 7.6 = 10 mμ*A*/ml; after addition of cysteine = 110 mμ*A*/ml. Thus a considerable activation of the enzyme EIII is brought about. After storing the extract in the cold over night some measurements were made as presented in Table 6.

Table 6. The influence of cysteine or calcium on the proteolytic activity of *Echinocardium* egg extract.

1.00 ml extract was incubated with 0.50 ml reagent for 30 minutes at 20° C, mixed with 3.00 ml gelatin and measured. pH 7.7.

Expt. No.	Reagent	mμ*A*/ml	Rel. activity
27	H_2O	8.5	1.00
28	Cysteine	35	4.10
29	$CaCl_2$ 0.03 M	3.7	0.44

It appears from Table 6 that cysteine activates the enzyme, although $^2/_3$ of the enzyme EIII has apparently been destroyed over night (at storage). Calcium ions lower the activity with 50 per cent. In Table 7 the experiment was repeated with fresh extract prepared in the same way. Strong activation with cysteine was then obtained. Also calcium produced a considerable increase in activity on this fresh extract. The effect of calcium may be attributed to the presence of the enzyme EII which may already have been destroyed in the stored extract.

Table 7. The influence of cysteine or calcium on the proteolytic activity of *Echinocardium* egg extract.

1.00 ml extract was incubated with 0.50 ml reagent for 30 minutes at 20° C, mixed with 3.00 ml gelatin and measured. pH 7.7.

Expt. No.	Reagent	mμ*A*/ml	Rel. activity
30	H_2O	5.3	1.0
31	Cysteine	76	14
32	$CaCl_2$ 0.03 M	14	2.6

Tables 8–10 present some experiments with alloxan, cysteine and periodate on extracts from *Echinocardium*. The aim of these experiments was to shed more light over the activation mechanism of the enzymes *in vitro*. The results

in Table 8 reveal that cysteine increases the activity at pH 7.7 approximately four times and alloxan approximately three times. These experiments were continued as shown in Table 9, where the preparation of the extract also includes freezing and thawing of the homogenate. The results in Table 9 show that compared to the original sample (expt. 36) approximately 75 per cent activation was obtained with cysteine or sodium periodate. With alloxan the activation was somewhat lower.

Table 8. The influence of cysteine or alloxan upon the proteolytic activity of *Echinocardium* egg extract.

Unfertilized eggs from *Echinocardium* were treated as usual, homogenized in a mixture of sea water and distilled water (1 : 1) and centrifuged. 1.00 ml extract was incubated with 0.50 ml reagent for 15 minutes at 20° C, mixed with 3.00 ml gelatin and measured. pH 7.7.

Expt. No.	Reagent	mμA/ml	Rel. activity
33	H_2O	3.7	1.00
34	Cysteine	15	4.10
35	Alloxan	10	2.70

For comparison similar experiments were carried out at pH 6.7 as recorded in Table 10. The results at this pH diverge in some respect from those in Table 5 *(Paracentrotus)* carried out at pH 7.7, in that cysteine alone has a stronger effect than alloxan alone. If cysteine is added to the extract after alloxan, the activating effect of the latter is considerably decreased. Sodium periodate has the same effect with or without addition of cysteine, and the activity values are of the same order of magnitude as those obtained by the influence of alloxan + cysteine. The results seem to indicate that cysteine activates EI and alloxan EII (by liberation from an inhibitor) and that in presence of both reagents part of one activated enzyme blocks or destroys the other.

As some *Echinocardium* extracts were rather viscous, the measuring of the proteolytic activity was difficult or impossible. Therefore various experiments were carried out to obtain extracts of normal viscosity (slightly higher than that of water). In some cases the homogenates were frozen and thawed, and

Table 9. Activation of an extract from *Echinocardium* eggs.

Unfertilized *Echinocardium* eggs were washed 3 times in sea water, homogenized in sea water + distilled water (1 : 1), frozen, thawed and centrifuged. 1.00 ml extract was incubated with 0.50 ml reagent for 30 minutes at 20° C, mixed with 3.00 ml gelatin and measured. pH 7.7.

Expt. No.	Reagent	mμA/ml	Rel. activity
36	H_2O	8.5	1.00
37	Cysteine	14	1.65
38	Alloxan	12	1.40
39	$NaIO_4$	14	1.65

Table 10. Activation of an extract from *Echinocardium* eggs.

The same extract as in Table 9. 1.00 ml extract was first incubated for 15 minutes at 20° C (pH 6.5) with 0.25 ml of reagent I and after that for further 15 minutes with reagent II. Then 3.00 ml gelatin was added und the activity measured at pH 6.7.

Expt. No.	Reagent I	Reagent II	mμA/ml	Rel. activity
40	H_2O	H_2O	13	1.00
41	Cysteine	H_2O	60	4.60
42	Alloxan, 0.01 M	H_2O	41	3.15
43	Alloxan, 0.01 M	Cysteine	29	2.20
44	$NaIO_4$, 0.005 M	H_2O	26	2.00
45	$NaIO_4$, 0.005 M	Cysteine	24	1.85

were thereafter exposed to extended centrifugation at 8 000 g. As a rule a non-viscous upper supernatant could be distinguished and separated from a lower, very viscous supernatant (mucous). The investigation of the nature of the viscous part was not considered to be within the scope of this paper. Experiments were made to reduce the viscosity by fractionating the solution with ammonium sulphate.

Unfertilized *Echinocardium* eggs were washed twice in sea water, homogenized in sea water + the equal volume distilled water and frozen. After thawing and storing for some hours in the cold the homogenate was centrifuged and a rather viscous solution was obtained as supernatant. A volume of this was kept in the cold with two volumes saturated ammonium sulphate solution, thus 65 per cent saturation. Precipitation was formed already at 30 per cent sat. but became more abundant when the whole amount of ammonium sulphate was added. The mixture was kept for three hours in the cold and then centrifuged. The precipitate was dissolved in dist. water, taken up to original volume and clarified by centrifugation. In Table 11 some activation experiments are shown with this purified extract of normal viscosity.

The results in Table 11 reveal that cysteine brings about good activation of the purified extract, as does also, although to a lesser extent, alloxan. In all samples, however, the activity started to decrease, declining to zero already after 30–40 minutes. The stability thus decreased considerably through the fractionation. The activating effect of alloxan may be attributed to the destruction of an inhibitor.

Table 11. Activation of a fractionated extract from *Echinocardium* eggs.

1.00 ml extract was incubated with 0.50 ml reagent for 30 minutes at 20° C, mixed with 3.00 ml gelatin and measured at pH 7.6.

Expt. No.	Reagent	mμA/ml
46	H_2O	20
47	Cysteine	115
48	$CaCl_2$, 0.03 M	25
49	Alloxan, 0.01 M	60

In Table 12 some activation experiments are recorded with extracts of *Echinocardium* eggs washed in isotonic medium and homogenized in potassium chloride. Unfertilized eggs were washed twice in sea water, three times in isotonic sodium chloride solution, and homogenized in 1 M KCl. The homogenate was kept in the cold over night and then centrifuged. The experimental values show that cysteine, as well as calcium or alloxan have an activating effect. In the calcium sample, however, the activity disappeared already after $^3/_4$ hours. This is a phenomenon which frequently occurs in connection with calcium.

Attempts to fractionate extracts from egg material washed in isotonic medium with ammonium sulphate failed, to the effect that the obtained extract was completely inactive at pH 6.7 as well as 7.8. Addition of cysteine, calcium or alloxan had no effect.

Table 12. Activation of extract from *Echinocardium* eggs washed in isotonic sodium chloride solution.

1.00 ml extract was incubated for 30 minutes at 20° C with 0.50 ml reagent, mixed with 3.00 ml gelatin and measured at pH 7.8.

Expt. No.	Reagent	mμA/ml	Rel. activity
50	H_2O	11	1.00
51	Cysteine	33	3.00
52	$CaCl_2$, 0.03 M	27	2.45
53	Alloxan	27	2.45

C. The activity of the neutral enzymes after fertilization (EI–EIII)

As it has been stated that the proteolytic activity at pH 7.8 is due to a special enzyme of SH type, named EIII by the author, the question arises whether this enzyme is like the SH enzyme EI activated some minutes after fertilization. The experiments presented in Tables 13 *(Paracentrotus)* and 14 *(Echinocardium)* aim at answering this question. The fact that addition of cysteine to extracts of fertilized eggs (3–5 min. stages) either has an activating effect or, if the activity is high, has no effect at all, seems to prove that the enzyme EII is not activated at this stage. Table 14 presents activation experiments with an extract from fertilized *Echinocardium* eggs treated in different ways.

Unfertilized *Paracentrotus* eggs were washed three times in sea water (0.6 ml 0.01 M $NaIO_4$ were added to 200 ml egg suspension). Some of this material was then fertilized. A membrane elevation of approx. 95 per cent was obtained. After slow centrifugation to half the volume the eggs were homogenized through glass filter, *c.* 8–10 minutes after the membrane elevation, and then centrifuged.

The residue of the egg suspension was not fertilized, but was otherwise treated exactly the same way. Nitrogen content: Unfertilized egg extract = 1.25; fertilized egg extracts = 0.92 mg/ml.

The results in Table 13 show that the activity at pH 6.7 in extracts from fertilized eggs is several times higher than in extracts from unfertilized eggs.

Table 13. The proteolytic activity measured at pH 6.7, 7.2 and 7.7 in extracts from unfertilized and fertilized eggs of *Paracentrotus.*

1.00 ml extract was mixed with 3.00 ml gelatin and measured.

Expt. No.	Stage	pH	mμA/ml	mμA/mg N	Rel. activity per mg N
54	Unfertilized	6.7	11	8.8	1.0
55	Fert. 8 minutes	6.7	60	65	7.4
56	Unfertilized	7.2	2	1.6	—
57	Fert. 8 minutes	7.2	2	2.2	—
58	Unfertilized	7.7	78	62	1.0
59	Fert. 10 minutes	7.7	150	160	2.6

At pH 7.2 the activity is very low and unchanged after fertilization (EII). At pH 7.7 the activity is high already in the extract from unfertilized eggs, but more than 50 per cent higher in extract from the fertilized eggs, all calculated on the activity per mg nitrogen.

Table 14 presents measurements of the activity in extracts from fertilized and unfertilized *Echinocardium* eggs where only 50 per cent of the eggs were fertilized. *Echinocardium* eggs were washed three times in sea water. An ordinary egg suspension was then made, part of which was fertilized. About 12 minutes after membrane elevation the egg material was homogenized and centrifuged. The unfertilized part of the egg suspension was treated in the same way. Nitrogen content: Unfert. extr. = 1.60 mg/ml, fert. extr. = 1.40 mg/ml.

As there was low or no activity in samples without cysteine, cysteine was added in the experiments recorded in Table 14. From this is evident that proteolytic activity occurs in both extracts at pH 6.8. The proteolytic activity is, however, twice as great in the material fertilized to 50 per cent, in both cases, as earlier mentioned, after addition of cysteine. Also at pH 7.6 the activity is considerably higher in the fertilized material.

To study an extract of fertilized *Echinocardium* eggs the experiments were carried out as recorded in Table 15. The eggs were washed three times in sea

Table 14. The proteolytic activity at pH 6.8 and 7.6 in extracts from unfertilized *Echinocardium* eggs.

100 ml extract was incubated with 0.50 ml cysteine for 20 minutes at 20° C, mixed with 3.00 ml gelatin and measured.

Expt. No.	Stage	pH	mμA/ml	mμA/mg N	Rel. activity per mg N
60	Unfertilized	6.8	25	16	1.0
61	Fert. 12 minutes	6.8	49	35	2.2
62	Unfertilized	7.6	32	20	1.0
63	Fert. 12 minutes	7.6	85	61	3.1

Table 15. Experiments on activation of extracts from fertilized *Echinocardium* eggs.

1.00 ml extract was incubated with 0.50 ml reagent for 30 minutes at 20° C, mixed with 3.00 ml gelatin and measured. Extract (A): one time frozen and thawed during extraction. Extract (B): the same procedure once repeated.

Expt. No.	Extract	Reagent	mg N/ml	pH	mμA/ml	mμA/mg N	Rel. activity
64	A	H_2O	1.4	6.8	17	12	1.0
65	A	Cysteine	1.4	6.8	17	12	1.0
66	A	$CaCl_2$, 0.03 M	1.4	6.8	6.0	4.3	0.36
67	A	Alloxan, 0.01 M	1.4	6.8	44	31	2.6
68	A	H_2O	1.4	7.6	17	12	1.0
69	A	Cysteine	1.4	7.6	28	20	1.7
70	B	H_2O	1.3	6.8	1.8	1.4	1.0
71	B	Cysteine	1.3	6.8	17	13	9.5
72	B	$CaCl_2$	1.3	6.8	6.2	4.8	3.4
73	B	Alloxan	1.3	6.8	0.2	0.2	0.1
74	B	H_2O	1.3	7.8	13	10	1.0
75	B	Cysteine	1.3	7.8	27	21	2.1
76	B	$CaCl_2$	1.3	7.8	3.2	2.5	0.3
77	B	Alloxan	1.3	7.8	62	48	4.8

water and fertilized, and *c.* 95 per cent membrane elevation was obtained. *C.* 7–8 minutes after the membrane elevation (10 minutes after the addition of spermatozoa) 1 volume slowly centrifuged egg material was mixed with 1 volume distilled water and homogenized. The homogenate was frozen, thawed and divided in two parts, (A) and (B).

(A) This part was centrifuged for 2 $^1/_2$ hours in the cold (in order to separate a rather viscous lower supernatant) at 7 000 g and measurements were carried out. (B) This part was again frozen, thawed and kept for three hours in the cold. As this solution was rather viscous only the upper non viscous supernatant could be separated also in this preparation, whereas the lower viscous opalescent part was thrown away. A study of the results in Table 15 reveals great similarities between the two extracts, yet with the exception for the effect of alloxan on the activity at pH 6.8. In the extract (A) frozen and thawed once, cysteine had no effect at pH 6.7, calcium lowered the activity to $^1/_3$ of the original value and alloxan increased the activity 2 $^1/_2$ times. At pH 7.6 cysteine doubles the activity. In the extract (B), twice frozen and thawed, the original activity is low but reaches the same value as in the extract (A) by influence of cysteine. Calcium gives the same activity value, which in this case implies an increase, whereas alloxan almost removes the existing low activity (EI). At pH 7.8 there is some original activity, which is doubled when adding cysteine and gets almost five times greater at the addition of alloxan. Calcium ions lower the activity considerably. It is of interest that the enzyme EI gets the same value in both extracts from cysteine, 17 and 27 mμA/ml at pH 6.7 and 7.6 respectively. The activity value 6 mμA/ml which is obtained by the influence of calcium may be due to the enzyme EII. In both extracts EIII seems to be active at pH 7.6–7.8.

D. The proteolytic activity in extracts from *Arbacia* and *Paracentrotus* eggs treated with periodate

RUNNSTRÖM and co-workers have shown that a brief treatment of the egg material with very weak periodate solution considerably facilitates the fertilization of eggs otherwise difficult to fertilize. Some investigations of homogenate from eggs treated with periodate but not fertilized have been carried out to this effect. The results are presented in Tables 16 and 17. In Table 18 experiments with alloxan and *Fucus* extracts are recorded which have been carried out on the same extract as in Table 17.

Unfertilized *Arbacia* eggs were washed three times in sea water. The diluted egg suspension was then divided in two parts. One part was kept as control, whereas 0.30 ml 0.01 M sodium periodate was added to 100 ml of the other part. The two suspensions were kept for 15 minutes at 18° C, and were then washed in sea water. After sedimentation the material was homogenized in c. three volumes of 1 M KCl and then centrifuged. Nitrogen content = 1.60 mg/ml in both extracts. In Table 16 the influence of periodate on *Arbacia* eggs is demonstrated, and it is evident that at pH 6.7 as well as at 7.7 the activity is somewhat lower in the periodate treated material. Cysteine has no effect on the activity, the lower value for A^{II} is fully normal, considering that two hours have passed. The differences in activity in extracts from treated and untreated egg material can possibly give a hint as to the effect of periodate treatment, but seem to be too inconsiderable to explain the fertilization promoting influence. In Table 17 experiments with extracts from *Paracentrotus* eggs are presented which have been treated in the same way.

Unfertilized *Paracentrotus* eggs were washed three times in sea water. Part of the diluted egg suspension was treated for 10 minutes at 20° C with sodium periodate (0.30 ml periodate 0.01 M + 100 ml egg suspension). Another part of the egg suspension was kept during the same time as control without reagent. After washing with sea water and sedimentation both suspensions were homogenized separately in three times the sedimented egg volume 1 M KCl and centrifuged. Nitrogen content: 1.6 mg/ml (control) and 1.5 mg/ml (periodate). The values of Table 17 are throughout of a higher order of magnitude than those recorded in Table 16 for *Arbacia* extracts.

Table 16. The proteolytic activity in extracts from unfertilized *Arbacia* eggs treated with periodate.

1.00 ml extract was mixed with 3.00 ml gelatin and measured (A^{I}). Two hours later 0.50 ml cysteine was added and the measurements continued (A^{II}).

Expt. No.	Treatment of eggs	pH	mμA^{I}/ml	mμA^{I}/mg N	mμA^{II}/ml	mμA^{II}/mg N	$A^{\mathrm{II}}/A^{\mathrm{I}}$
78	Untreated	6.7	19	12	13	8.1	0.68
79	Periodate	6.7	16	10	11	6.9	0.69
80	Untreated	7.7	26	16	22	14	0.85
81	Periodate	7.7	16	10	10	6.3	0.63

Table 17. The proteolytic activity in extracts from unfertilized *Paracentrotus* eggs treated with periodate.

1.00 ml extract was mixed with 3.00 ml gelatin and measured (A^{I}). 1 $^1/_2$ hours later 0.50 ml cysteine was added and the measurements continued (A^{II}).

Expt. No.	Treatment of eggs	pH	mμA^{I}/ml	mμA^{I}/mg N	mμA^{II}/ml	mμA^{II}/mg N	A^{II}/A^{I}
82	Untreated	6.6	200	125	110	69	0.55
83	Periodate	6.6	105	70	27	18	0.26
84	Untreated	7.7	100	63	140	88	1.40
85	Periodate	7.7	45	30	36	24	0.80

The former values (Table 17) are abnormally high for extracts from unfertilized eggs but a study of the influence of periodate still ought to be of interest. The original activity at pH 6.6 and 7.7 is thus twice as high in untreated as in periodate treated material. The effect of cysteine is noteworthy. At pH 6.6 cysteine lowers the activity in untreated material, but still more in periodate treated material. The lower activity in periodate material seems to indicate a lower content of the enzyme EI, and the inactivating effect of cysteine indicates the presence of EII. At pH 7.7 cysteine has an activating influence on untreated material, which speaks in favour of the occurrence of enzyme EIII. In the periodate treated material, however, the activity is lower but more stable and is not influenced by cysteine. This may be due to the removal of an inhibitor through the periodate treatment. Corresponding values in Table 16 also speak in favour of a stabilization of the enzyme activity in the periodate treated material.

In Table 18 experiments on the two extracts studied in Table 17 are presented. The influence of alloxan (fresh solutions) and of preparations from *Fucus* extracts[1] is studied in this table. Some factor in extracts of *Fucus vesiculosus* has proved to inhibit fertilization (cf. WICKLUND (10)). It is by the physiologists called FeInh (Fu), that is, Fertilization Inhibiting Factor from *Fucus.* Alloxan, which was first studied by this author (9) and later by WICKLUND, (10) lames the spermatozoa at the same time as it seems to have an inhibiting influence on the cleavage of the egg (10). As the experiments in Table 18 reveal, alloxan has only a slightly activating effect at pH 6.6, which may be due to the presence of EII. At pH 7.7 in this extract, however, alloxan increases the activity considerably. The enzyme EIII is apparently activated. The fucus preparation (Sup C_4I) lowers the activity considerably at both pH values. EII may reach an optimum at pH 7.2–7.3.

For technical reasons measurements could not be carried out with the periodate treated extract at pH 7.7. The original activity in this extract at pH 6.6 is considerably lower than that in the control extract and cysteine lowers the activity, which indicates that part of it comes from enzyme EII. Alloxan has an activating effect and the fucus preparation (Sup C_4I) lowers the activity with $^2/_3$ as it also did at the same pH in the control extract. The enzyme activity at pH 7.3 is greater than that at pH 6.6 (EII).

---

[1] Preparations from extracts from *Fucus vesiculosus* were kindly supplied by Dr. E. VASSEUR which is herewith gratefully acknowledged.

As a conclusion it may be said that a comparison between extracts from periodate treated and untreated eggs shows that the enzyme activity is higher in the untreated material and also shows different content of the three enzymes. In the periodate treated material there seems to be more EII at the expense of EI and EIII than in the untreated material which appears natural considering that EI and EIII are defined as thiol enzymes. It seems as if alloxan increases the activity of EII and EIII and as if the fucus preparations lowers the activity of EII and EIII.

Table 18. Experiments on activation of extracts from unfertilized *Paracentrotus* eggs treated with periodate.

1.00 ml extract was incubated with 0.50 ml reagent for 30 minutes at 20° C, mixed with 3.00 ml gelatin and measured.

Expt. No.	Treatment of eggs	Reagent	pH	$m\mu A$/ml	$m\mu A$/mg N	Rel. activity
86	Untreated control	H_2O	6.6	220	135	1.0
87	,, ,,	Alloxan, 0.01 M	6.6	235	145	1.1
88	,, ,,	Fucus: SupC$_4$I 0.1 %	6.6	72	45	0.33
89	,, ,,	H_2O	7.3	135	85	—
90	,, ,,	H_2O	7.7	115	72	1.0
91	,, ,,	SupC$_4$I 0.1 %	7.7	62	39	0.54
92	,, ,,	Alloxan	7.7	200	125	1.7
93	Periodate	H_2O	6.6	38	25	1.0
94	,,	Alloxan	6.6	57	38	1.5
95	,,	SupC$_4$I 0.1 %	6.6	13	8.7	0.34
96	,,	Cysteine	6.6	29	19	0.76
97	,,	H_2O	7.3	68	45	—

E. The influence of isotonic (calcium free) washing of the egg material on the proteolytic enzyme activity

It is of great interest to shed more light over the effect of different treatments of the egg material before homogenization and extraction upon the enzyme activity. It seems probable that the enzymes active at pH 7 are influenced by this treatment. Previous papers have elucidated this question to some extent. It is evident from Tables 12, 13, 15, 20 and 25 in an earlier paper (2), as well as from Tables 1 and 9 in another paper (3), that washing of the egg material with isotonic sodium chloride preferably gives the enzyme EI. One exception is found in Table 16 (2) which reveals very high original activity at pH 6.6. This activity is destroyed by cysteine to 98 per cent and is attributed to the enzyme EII according to the definition of this enzyme. The experiments have been continued and the results are presented below and in Tables 19–21.

Unfertilized eggs from *Arbacia* were washed three times in sea water and twice in isotonic sodium chloride solution. They were homogenized in isotonic solution and centrifuged. The supernatant was called (A). N = 1.30 mg/ml. The sediment was suspended in 1 M KCl, kept for half an hour in the cold and centrifuged (B). 1.00 ml extract (A) was mixed with 3.00 ml gelatin and the activity was measured. After two hours 0.50 ml cysteine was added and the measurements continued. At pH 6.6 the activity was 35 mμ*A*/ml but disappeared after one hour. After the addition of cysteine a slight reactivation occurred up to 23 mμ*A* ml. At pH 7.7 the activity was 4.2 mμ*A*/ml. After the addition of cysteine no reactivation was obtained at this pH. The results indicate that only the enzyme EI was obtained. The sediment (B) extracted in KCl from the isotonic sodium chloride extraction was inactive at the two pH values and cysteine had no effect. At the same treatment of *Paracentrotus* the value 59 mμ*A*/ml was obtained in the sodium chloride extract at pH 6.6 and 32 mμ*A*/ml at pH 7.7. After one hour cysteine was added and the activity ceased. This indicates that the enzyme EII was obtained.

The scope of the experiments in Table 19 was to compare the influence of certain substances on extracts from eggs washed in isotonic solution which had been extracted otherwise than in the two first-mentioned cases.

Unfertilized eggs from *Paracentrotus* were washed in sea water and three times in isotonic sodium chloride solution. The sedimented eggs were then homogenized in the same volume (= its own) distilled water and centrifuged. The clear suspension was designated I. Nitrogen content = 2.10 mg/ml. The sediment was suspended in the same volume 1 M KCl, kept for one hour in the cold and centrifuged. The clear suspension was designated II. Nitrogen content = 1.3 mg/ml.

As is evident from Table 19 strong activation is obtained with calcium at pH 6.6, whereas cysteine lowers the activity to *c.* $^{1}/_{4}$. This indicates a higher content of the enzyme EII than EI in extract I. After storage in the cold for

Table 19. Experiments on activation of extracts from unfertilized *Paracentrotus* eggs washed in isotonic sodium chloride solution.

1.00 ml extract was incubated with 0.50 ml reagent for 30 minutes at 20° C, mixed with 3.00 ml gelatin and measured.

Expt. No.	Extract	Reagent	pH	mμ*A*/ml	mμ*A*/mg N	Rel. activity
98	I (dilut. NaCl)	H_2O	6.6	99	47	1.0
99	I	$CaCl_2$, 0.03 M	6.6	175	83	1.8
100	I	Cysteine	6.6	75	36	0.76
101	I	H_2O	7.7	235	110	1.0
102	I	$CaCl_2$	7.7	270	130	1.2
103	I	Cysteine	7.7	45	21	0.19
104	I	Alloxan, 0.01 M	7.7	375	180	1.6
105	II (1 M KCl)	H_2O	7.7	27	21	1.0
106	II	$CaCl_2$, 0.03 M	7.7	34	26	1.3
107	II	Cysteine	7.7	36	28	1.3
108	I (extract stored 2 days at +3°)	H_2O	6.6	130	62	—
109	I (do)	H_2O	7.7	130 (instable)	62	1.0
110	I (do)	Alloxan, 0.01 M	7.7	225 (stable)	105	1.7

two days, 50 per cent higher activity (expt. 108) was brought about than in the original sample (expt. 98) at pH 6.6. EI or an inhibitor blocking EII may have been destroyed during storage. At pH 7.7 a weak activating influence is obtained by calcium, cysteine lowers the activity with 80 per cent and alloxan increases it with 70 per cent. Also at this pH the enzyme EII predominates. After two days' storage in the cold the activity in the distilled water extract (the original activity) at pH 7.7 decreased with about 50 per cent of the original activity (expt. 101) and decreased more rapidly during the measurement (expt. 109). Alloxan increases the activity to about the original value and has besides a stabilizing effect. At pH 7.7 an inhibitor which is counteracted by alloxan seems to block EII. At pH 6.6, however, the effect of an inhibiting factor seems to have decreased spontaneously during the storage of the extract.

The potassium chloride extract from the sediment has low activity at pH 7.7, but increases with about $^1/_3$ from calcium as well as from cysteine.

Some further experiments with extracts from *Paracentrotus* eggs are shown in Tables 20 and 21. These experiments aim at studying the difference in activity at pH 6.6 and 7.7 between extracts from eggs, either washed in sea water or in isotonic sodium chloride solution and homogenized in 1 M KCl.

Unfertilized eggs from *Paracentrotus* were washed twice in sea water and the sedimented egg material was then divided in two parts, (A) and (B). (A) was washed in a big volume of sea water and (B) was washed in a great volume of isotonic sodium chloride. After sedimentation the eggs were suspended in equal volumes 1 M KCl, homogenized and centrifuged. Nitrogen (A) 1.35 mg/ml, and (B) 1.50 mg/ml. It is evident from Table 20 that cysteine increases the activity at pH 6.6 to a higher extent in the extract from eggs washed in sea water (A) than in the extract from eggs washed in isotonic medium (B). There is thus a greater content of the enzyme EI in (A) than in (B).

Table 20. The influence of cysteine upon the proteolytic activity of extracts from *Paracentrotus* eggs washed in different solutions.

1.00 ml extract was mixed with 3.00 ml gelatin and measured (A^{I}). One hour later 0.50 ml cysteine was added and the measurement continued (A^{II}). pH 6.6.

Expt. No.	Washing medium	mμA^{I}/ml	mμA^{I}/mg N	mμA^{II}/ml	mμA^{II}/mg N
111	Sea water	29	21	100	74
112	Isotonic NaCl	25	17	44	29

In Table 21 some other experiments with these extracts are recorded. The original activity at pH 6.6 in extract (A) was 29 mμA/ml in Table 20. In Table 21 where the sample was first incubated at 20° C for 30 minutes it amounts to as much as 66 mμA/ml. The *Fucus* preparations (Sup C_4I) had to influence on this extract at pH 6.6, whereas alloxan removes about 85 per cent of the activity. The inactivating effect of alloxan on the enzyme EI is here clearly noticeable. The influence of calcium was studied at pH 7.7 and it turned out that the activity increased with about 45 per cent in extract from eggs washed in sea water, whereas calcium was inactivated with about 25 per cent

Table 21. Experiments on activation of extracts from *Paracentrotus* eggs.

1.00 ml extract was incubated with 0.50 ml reagent for 30 minutes at 20° C, mixed with 3.00 ml gelatin and measured.

Expt. No.	Washing medium	Reagent	pH	mμ*A*/ml	mμ*A*/mg N
113	Sea water	H_2O	6.6	66	49
114	”	Fucus: $SupC_4I$ 0.1 %	6.6	66	49
115	”	Alloxan, 0.01 M	6.6	10	7.4
116	”	H_2O	7.7	29	21
117	”	$CaCl_2$ 0.03 M	7.7	42	31
118	Isotonic NaCl	H_2O	7.7	51	34
119	” ”	$CaCl_2$ 0.03 M	7.7	37	25

in extract from eggs washed in isotonic medium. Approximately the same values were reached in both cases.

The result of the influence of cysteine on extracts of *Paracentrotus* eggs washed and homogenized in sea water is as follows. 1.00 ml extracts was mixed with 3.00 ml gelatin and measured. After one hour 0.50 ml cysteine was added and the measurements continued. At pH 6.6 the activity was 34 before and 43 mμ*A*/ml after addition of cysteine, which seems to indicate the presence of the enzyme EI. At pH 7.7 the activity before the addition of cysteine was 130 and afterwards 120 mμ*A*/ml, which shows the existence of EIII with optimal original activity.

F. Investigations on the proteolytic activity of some jelly coat solutions

In an earlier paper (1) proteolytic activity was reported in solutions of jelly coats from *Arbacia, Paracentrotus,* and *Echinus esculentus.* The jelly coats were obtained through acidification of sea water (pH 5.5) and precipitation with ethanol. The precipitate was dissolved in a small amount of distilled water, dialyzed against running tap water and lyophilized. From these dried preparations extracts were made which possessed a certain activity when gelatin or hemoglobin were used as substrate. The results of a series of these investigations were recorded in Table III of the above-mentioned paper (1).

Since these results were published, experiments have been carried out the results of which seem to indicate that the proteolytic activity found in jelly coat solutions is due to enzymes in the egg. One example of a similar series of experiments is shown in Table 22. The experiments were carried out on one of the preparations examined in Table III of the above-mentioned paper (1).

A small amount (0.125 g) of an ethanol precipitated and lyophilized jelly coat preparation from *Echinus* was extracted with 25 ml synthesized sea water in the cold for three hours and then centrifuged. Nitrogen content = 0.012 mg/ml. The pH value of the jelly coat solution was 7.4. The results reveal strong activation of the enzyme through periodate, as well as activating effect of iodoacetamide. The inactivating effect of cysteine and the earlier mentioned

Table 22. Some experiments with a jelly coat solution possessing proteolytic activity.

1.00 ml jelly coat solution was incubated with 0.50 ml reagent for 1 hour at 20° C, mixed with 3.00 ml gelatin and measured at pH 6.8.

Expt. No.	Reagent	mμ*A*/ml	mμ*A*/mg N	Rel. activity
120	H_2O	6.3	525	1.0
121	Cysteine	1.8	150	0.3
122	$NaIO_4$, 0.01 M	34	2850	5.4
123	Iodoaceteamide, 0.05 M	12	1000	1.9

fact (cf. Table 24 (2)) that enzyme EII can easily diffuse from the egg when washed in isotonic solution support the concept that the activity of the jelly coat solution may be assigned to the enzyme EII. The activating effect of periodate may mainly be due to its destructive effect on an inhibitor, which blocks part of EII.

The diffusion of EII may prove that this enzyme is localized in the outer layer of the egg. It was already suspected earlier (1) that proteolytic activity did not occur in the jelly coat. Continued observations and studies have confirmed this assumption. It thus turned out that enzymes sometimes leaked out into the surrounding medium during washing of the egg material. This happened in isotonic as well as in hypo- or hypertonic saline solutions, and in sea water. It seems probable that the enzymatic activity of some jelly coat solutions is due to enzymes leaking out from the egg and precipitating with the jelly coats which were brought into solution. This concept is confirmed by the fact that the proteolytically active jelly coat preparations examined have in most cases been slightly brown or pink coloured. This may be attributed to careless removal of the jelly coats.

Table 23 presents an experiment with a solution of jelly coats from *Paracentrotus* eggs cautiously brought into solution and of egg extracts from the same eggs deprived of their jelly coats. Unfertilized *Paracentrotus* eggs were washed twice in sea water and the egg material was divided in two parts. One part was homogenized in twice its volume sea water and centrifuged. In the other part the jelly coats were removed. To 50 ml egg suspension 3 ml 0.1 *M* HCl was added dropwise and stirred. The pH value then reached 5.5. The eggs, now freed from their jelly coats, spontaneously sedimented, and the pH value was restored. The jelly coat solution was then separated and centrifuged. The control of the material showed intact jelly coat free eggs. It is evident from Table 23 that the jelly coat solution completely lacks proteolytic activity with as well as without cysteine both at pH 6.7 and 7.6. The low activity in the egg extract at pH 6.6 which increases threefold after addition of cysteine proves that the egg material was normal. Also in fertilization experiments, the eggs behaved normally. At pH 7.5 the activity of the extract is rather strong and is not further increased by addition of cysteine. The data presented should give sufficient evidence that the jelly coat solutions do not possess proteolytic activity.

Table 23. Determination of the proteolytic activity in a fresh jelly coat solution and in the extract from the corresponding egg material.

1.00 ml extract or jelly coat solution was mixed with 3.00 ml gelatin and measured (A^{I}). 90 minutes later 0.50 ml cysteine was added and the measurements continued (A^{II}).

Expt. No.	Solution	pH	mμA^{I}/ml	mμA^{II}/ml
124	Egg extract	6.6	10	29
125	,,	7.5	105	95
126	Jelly coat	6.6	0	0
127	,,	7.6	0	0

II. The hatching enzyme

Earlier investigations by Ishida (4), Kopac (5) and Sugawara (6) on the sea urchin species *Strongylocentrotus pulcherrimus* and *Arbacia punctulata* have revealed that a concentrated medium from the hatching blastula stage contains an enzyme able to dissolve the fertilization membrane. In order to compare this enzyme with those studied by this author similar experiments were carried out on *Paracentrotus lividus.* The results of these experiments are presented in Tables 24–26.

Table 24. The proteolytic activity of extracts from hatching blastula of *Paracentrotus* eggs.

1.00 ml extract was incubated with 0.50 ml reagent for 30 minutes at 20° C, mixed with 3.00 ml gelatin and measured.

Expt. No.	Preparation	Reagent	pH	mμA/ml	mμA/mg N
128	HEIA	H_2O	6.7	17	90
129	,,	Cysteine	6.7	20	105
130	,,	H_2O	7.7	22	120
131	,,	Cysteine	7.7	25	130
132	,,	H_2O	8.9	67	355
133	HEIB	H_2O	8.9	49	410
134	,,	Alloxan, 0.01 M	8.9	20	170
135	,,	$CaCl_2$, 0.03 M	8.9	6.9	58

Unfertilized *Paracentrotus* eggs were as usual washed three times in sea water. Some of the unfertilized material was separated for direct homogenization as a control and then centrifuged. The supernatant is called NFControl. Nitrogen content: 1.30 mg/ml. Another part of the egg material was fertilized (*c.* 98 per cent membrane elevation) and the eggs were allowed to develop at 18° C in shallow discs which were rocked. After 12 hours 50 per cent of the eggs were hatched. The material was concentrated by means of slow centrifugation and the supernatant medium was collected. This is designated HEMed. The sedimented egg material was divided and frozen in two lots, (A) and (B).

Table 25. Proteolytic activity of the hatching blastula from *Paracentrotus.*

1.00 ml extract was mixed with 3.00 ml gelatin and measured.

Expt. No.	Extract	mg N/ml	pH	mμ*A*/ml	mμ*A*/mg N
136	HEII	1.40	6.7	53	38
137	„	1.40	7.7	46	33
138	„	1.40	8.7	25	18
139	HEIIR	0.84	6.7	4.2	5.0
140	„	0.84	7.7	4.8	5.7
141	„	0.84	8.8	7.8	9.3
142	HEIIF	1.55	6.7	9.0	5.8
143	„	1.55	7.7	10	6.7
144	„	1.55	8.8	11	7.1

After thawing these lots were centrifuged. The supernatants are designated HEIA and HEIB. Nitrogen content = 0.19 mg/ml and 0.12 mg/ml. Measurements revealed no activity in the control extract from unfertilized eggs at pH values 6.7, 7.7 and 8.9. Nor was the medium in which the eggs developed (HEMed) enzymatically active (cf. Ishida and Sugawara). During biological experiments (Wicklund) no influence of the fertilization membrane *in vivo* was obtained. In extracts from hatching blastula (HEIA), however, activity occurred at the three pH values and was about three times stronger at pH 8.9 than at pH 6.7 or 7.7. This agrees with the optimum value at pH 8.5–9.5 as indicated by Sugawara (6). Biological experiments carried out analogously (Wicklund) revealed strong dissolving effect on the fertilization membrane. Cysteine had a slightly activating effect at pH 6.7 and 7.7. Alloxan had an inactivating effect on the hatching enzyme and so had calcium. The latter (cf. Sugawara) may be due to too high a concentration of calcium. As mentioned above the extract from unfertilized eggs was inactive at pH 6.6 as well as at pH 7.7 and 8.9. Therefore, no comparison could be made as to the activity of the hatching enzyme at the same pH values. Thus new homogenates from unfertilized eggs were prepared until a homogenate active at pH 6.7 was obtained. This homogenate was also measured at pH 7.7 and 8.8. The activity was 55 mμ*A*/ml at pH 6.7, about twice as high (100 mμ*A*/ml) at pH 7.7, but less than $^1/_{10}$ at pH 8.8 (4 mμ*A*/ml). The difference is clearly shown as to the activity distribution of this extract at different pH values, compared to the activity values of the hatching enzyme recorded in Tables 24 and 25. Table 25 gives the measurements of other extracts from hatching blastula.

Unfertilized *Paracentrotus* eggs were washed in sea water and fertilized (98 per cent). The eggs were allowed to develop into hatching blastula. The egg suspension was then concentrated and homogenized through glass filter. Part of the homogenized material was separated and centrifuged. The supernatant was called HEII, nitrogen content = 1.40 mg/ml. The sediment was suspended in distilled water, frozen, thawed and kept for two hours in the cold and centrifuged. The supernatant was called HEIIR. Nitrogen content = 0.84 mg/ml. Another part of the homogenized egg material was frozen, thawed, kept in the cold for two hours and centrifuged. The supernatant is called HEIIF, nitrogen content = 1.55 mg/ml. Table 25 shows that the activity is rather low over all

Table 26. The influence of cysteine upon the activity of the hatching enzyme from *Paracentrotus.*

1.00 ml HEII (see Table 25) stored at +3° for 2 days was mixed with 3.00 ml gelatin and measured (A^{I}). Three hours later 0.50 ml cysteine was added and the measurements continued (A^{II}).

Expt. No.	pH	mμA^{I}/ml	mμA^{I}/mg N	mμA^{II}/ml	mμA^{II}/mg N	A^{II}/A^{I}
145	6.7	5.4	3.9	13	9.3	2.4
146	7.7	8.0	5.7	12	8.6	1.5
147	8.7	14	10	13	9.3	0.9

and that it does not increase towards pH 8.8 in the centrifuged homogenate HEII obtained without freezing and thawing, but is in this extract strongest at pH 6.7. In the extract HEIIR obtained from the sedimented residue by freezing and thawing, the activity is, however, greatest at pH 8.8. Also in the extract HEIIF obtained by freezing and thawing of the total homogenate the activity tends to increase towards the alkaline pH.

In Table 26 some experiments with HEII are recorded with and without addition of cysteine. At pH 6.7 and 7.7 cysteine has an activating effect (EI and EIII, respectively). At pH 8.7 cysteine has no effect. Compared to measurements carried out two days previously (expts. 136 and 138) the activity at pH 6.7 in Table 26 is only *c.* 10 per cent, at pH 7.7 *c.* 20 per cent and at pH 8.7 *c.* 50 per cent of the original activity, as shown in Table 25. This proves that the hatching enzyme is more stable than enzymes EI and EIII.

Survey and discussion

A survey of the role of the proteolytic enzymes at fertilization

In extracts from unfertilized sea urchin eggs various proteolytic enzymes have been found, as previously reported. The first investigations revealed the presence of an enzyme active between pH 6 and 7 which was activated *in vivo* at fertilization, i.e. during the first minutes after membrane elevation. This enzyme which was also activated *in vitro* with cysteine was called EI. Sometimes there was relatively strong, but transient activity in centrifuged fresh homogenates of unfertilized eggs around neutral pH, which almost entirely disappeared upon addition of cysteine. This enzyme called EII was also obtained through extraction of lyophilized egg material including freezing and thawing, although it was then considerably weaker.

pH-activity curves determined by means of formol titration on centrifuged homogenates from fresh eggs, however, revealed activity at pH 4, and two optima around pH 7, *viz.* at pH 6.7 and at pH 7.8 (Fig. 1). The problem then arose about the relationship of EI and EII to the two last-mentioned pH optima. Continued research has indicated that EI has its optimum at 6.7 whereas the enzyme with its optimum at pH 7.8 can be activated by cysteine and is consequently of thiol type like EI. It is called EIII. The activity optimum of EII is probably around pH 7.2. Besides there is a proteolytic enzyme with optimal activity at pH 4.3–4.5 in all egg material. It is of the type cathepsin II, i.e. dependent of thiol groups for its function. It breaks down benzoylarginine amide but remains unchanged at fertilization. The pH optima of the different egg enzymes are schematically shown in Fig. 2. It must here be pointed out that the pH activity curves for enzyme EII and the hatching enzyme also studied are only outlined.[1] The role at fertilization of the enzymes EI, EII, and EIII, the activity optima of which are within the physiological pH interval, will here be discussed. Fig. 3 shows the probable appearance of these enzymes at fertilization according to the author's opinion. The following division corresponds to that in Fig. 3. In normal cases, i.e. when the eggs are easily fertilized, without treatment, the following scheme might be relevant.

A. In the unfertilized egg the enzymes EI, EII, and EIII occur in a non-active state.

B. In the moment when the fertilizing spermatozoon penetrates the egg a change of the cortical granules sets in. Calcium very likely plays a great role in this process. The activation of enzyme EII is now initiated.

C. Membrane elevation then takes place indicating that fertilization has occurred. At the same time the activity of EII reaches its optimum.

D. Immediately in connection with the high activity of EII, activation of EI and EIII occurs, as has been stated in extracts or homogenates from eggs collected some minutes after the membrane elevation. Processes may now occur by the influence of EII, through which SH groups are uncovered and EI and EIII rendered active (cf. HULTIN (11)). The activity of EII then seems to decrease rapidly. Inhibitors probably also participate in these enzymatic processes. It has so far not been possible to isolate these inhibitors which, however, are very likely to exist.

E. Some minutes later, i.e. more than ten minutes after the membrane elevation, the activity of EI and EIII also approach low values.

In the following some of the results and observations which speak in favour of the enzymatic processes outlined above will be reported.

In extracts from lyophilized unfertilized eggs (normal material) or in fresh homogenates of such material the proteolytic activity is in general low or non existing around neutral pH. The activity can, however, be considerably increased through the influence of cysteine at pH 6.7 and 7.8, which proves the occurrence of EI and EIII in a non active state. The activity in extracts from lyophilized eggs can, of course, never reach as high values as in centrifuged fresh homogenates. It should also be pointed out that if the eggs are unripe, overripe or on the whole difficult to fertilize, the above presented data are not valid.

RUNNSTRÖM and KRISZAT (12, 13, 14) found that eggs treated with SH-inhibitors at fertilization with normal sperm have normal membrane elevation, but that the eggs do not later develop normally. Eggs fertilized in the normal way also exhibit increased susceptibility to SH inhibitors during the first half hour after fertilization, which is in agreement with the appearance of EI and EIII.

---

[1] Proteolytic enzymes may have different pH activity curves for different substrates, see NORTHROP, J. H., KUNITZ, M., and HERRIOTT, R. M., Crystalline Enzymes, New York, 1948.

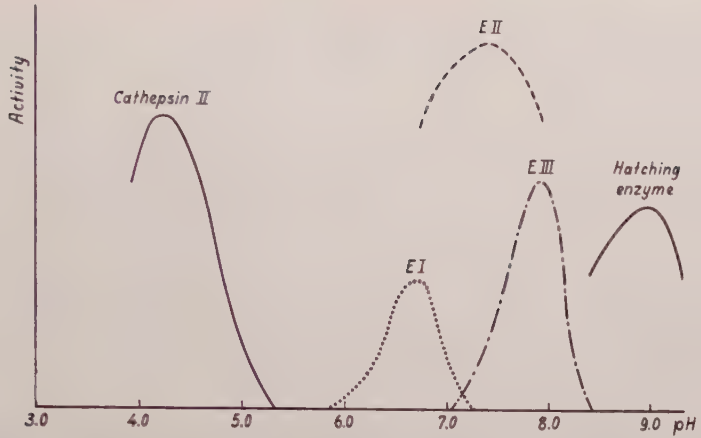


Fig. 2. Diagram showing pH optima of proteolytic enzymes in homogenates of unfertilized sea urchin eggs. It has not been possible to determine the pH optimum of enzyme EII in details, cf. text. The pH optimum of the hatching enzyme is shown for comparison.

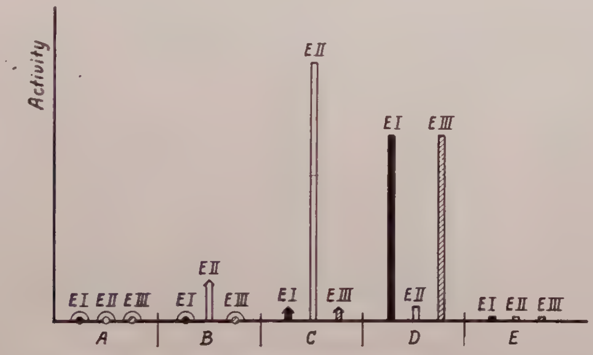


Fig. 3. A scheme showing the activity of proteolytic enzymes postulated in intact sea urchin eggs before and after fertilization.

A: Before fertilization.
B: At fertilization.
C: At membrane elevation.
D: Immediately after membrane elevation.
E: Ten minutes or more after membrane elevation.

The present study has shown that eggs difficult to fertilize may possess a higher original activity of EI and EIII. Egg material of this kind washed before fertilization with sea water containing sodium periodate in very low concentration proved to be much easier to fertilize. An investigation of the extracts revealed that the treatment caused lowered EI and EIII activity which could, however, be increased again with cysteine *in vitro*. Earlier investigations of the enzyme activity in extracts of eggs with abnormal development revealed an abnormal state with regard to the activity of EI (1). The activity was stronger before than after fertilization. There may, however, only be an assumed or indirect relation between the fertilization-promoting influence of periodate and its activity-lowering effect on the thiol enzymes EI and EIII. It is more likely that the physiological effect of periodate is, at least predominantly, caused by its influence on one or several inhibiting factors in the egg. This problem will be further discussed.

Calcium which has proved to be necessary for normal fertilization and the subsequent development, probably exerts its influence through enzyme EII which might be associated with the membrane elevation. In absence of calcium no normal membrane elevation takes place and the cortical granules behave otherwise than normally, cf. RUNNSTRÖM (15) and ENDO (16).

When the egg material is washed in isotonic or hypotonic saline solution the enzyme EII sometimes diffuses into the medium. This supports the view that EII is localized in cortex. From Table 22 (2) it is, however, evident that also enzyme EI may leak out into the medium. However, this only occurs exceptionally.

Several facts support the existence of an EII inhibitor in the egg. Treatment of normal egg material with sodium periodate and subsequent so called hypertonicity test, causes membrane elevation, i.e. activation of the egg. Periodate thus seems to have a destructive effect on some factor blocking EII. For reasons to be discussed later this inhibitor seems to be of heparin type. The fact that heparin has proved to inhibit EII strongly supports this concept, as does also the following. It has earlier been reported that jelly coats which have been removed from the eggs, precipitated with ethanol and again dissolved, exhibited a certain proteolytic activity. It could later be stated that this activity originated from the egg and that it was most probably the enzyme EII that leaked out. The proteolytic activity which sometimes accompanies jelly coat solutions was also shown to increase by the influence of periodate. This was most likely brought about by the destruction of some inhibitor of carbohydrate nature. It is evident from experiments with fresh alloxan solutions as described in this paper that enzyme EIII is strongly activated through this agent. So is also EII, whereas alloxan makes the activity of EI decrease. The activating effect is probably caused by alloxan's destruction of the inhibitor, but perhaps also to some degree to its destruction of enzyme EI.

Certain phenomena occurring during treatment of the egg with clupein may be related to the appearance of proteolytic enzymes. Clupein caused activation of the egg (nucleus migration toward the center and monaster formation), but the membrane elevation does not occur normally, the membrane is granular and low (cf. (17)). Recent experiments (18) reveal that clupein promotes higher activity in EIII, i.e. the SH dependent enzyme with its optimum at pH 7.8. The "membrane elevating" enzyme EII is, however, not influenced by clupein, but is probably indirectly injured either by the activating enzyme EIII or by influence on the cortical granules. This may explain the appearance of the abnormal membrane.

Ribonuclease has been found to increase the proteolytic activity (19) in extracts from lyophilized eggs or frozen egg homogenates at pH 6.7 and 7.8. Several other species were investigated (18) and extractions were carried out in different media and under different conditions. It then turned out that the liberation of the proteolytic enzymes upon treatment may vary considerably with the experimental conditions. This will, however, not be discussed in this paper.

Inhibiting and activating factors

In the present paper the occurrence and appearance of three enzymes active near neutral pH have been demonstrated and investigated. EI and EIII are activated by SH groups, whereas EII is inactivated by these. Various experiments give evidence that even certain inhibitors blocking EII and EIII are thiol compounds. Whether EII and EIII are blocked by the same inhibitor is difficult to decide. Regarding the nature of the inhibiting factors the following may be reviewed. The activation of enzyme EIII with clupein is evidence of the occurrence of acid groups in the inhibitor. The below discussed results of the treatment with alloxan point to the occurrence of functional SH groups. The periodate experiments discussed further below and some experiments briefly reported speak in favour of the existence of a heparin-like inhibitor. HARDING (20) found that sodium periodate removed the inhibiting effect of heparin on fertilization and in the present work data are presented to the effect that heparin inactivates enzyme EII, although it does not influence enzyme EI, as pointed out earlier. A comparison with the activity of ribonuclease may here be of interest.

ROTH (21) found that sulphydryl reagents increased the activity of ribonuclease in liver and assumed that this was due to an inhibitor formed by heparin or a heparin-lipoprotein complex, both of which contained cysteine. It has been shown that heparin is able to inactivate ribonuclease from sea urchin eggs, see LINDVALL and CARSJÖ (22).

The inactivating effect of cysteine on enzyme EII has been discussed in previous papers (2, 3). The inhibiting effect of cysteine in presence of oxygen on tissue proteases described by IRWING, FRUTON and BERGMANN (23) may here be of particular interest. HANSON and SMITH (24) report inactivating effect of cysteine on the enzyme carnosinas. The investigations by HERBERTS (25) of the proteolytic activity (at pH 7.2) “in tissue extracts from shocked organs after anaphylactic shock in guinea pigs'' are also of great interest. HERBERTS found that cysteine in a concentration of 0.02 per cent, or lower, increased the proteolytic activity. Continued experiments revealed, however, that higher concentrations of cysteine had an inactivating effect. Glutathion and a number of thiol compounds in far lower concentrations than cysteine also had an inhibiting effect. A system inactivated by some other thiol compounds than cysteine was completely reactivated by alloxan (0.05 per cent) and also by cysteine (0.02 per cent). Some results in the present paper might also be explained by similar phenomena, although this author has not succeeded in obtaining different effects with various concentrations of cysteine.

Alloxan, which is known to cause diabetes, has also been used as inhibitor of thiol enzymes like ureas (26), cholinesterase (27) and phosphorylase (28). Alloxan is found to have an activating effect upon enzymes EII and EIII (see Tables 5, 8–12, 15, 18, and 19). This effect is either brought about by oxidation of an inhibitor requiring intact SH groups for its function, which are in that case easier to oxidize than enzyme EIII, or perhaps rather by the formation of an addition compound with this inhibitor. The latter assumption seems more probable (cf. (29)). Enzyme EI is inactivated by alloxan (Tables 15 and 21), although the effect is mostly masked by the activating effect mentioned above.

The inactivating effect is very likely brought about by alloxan's oxidation of SH groups in EI.

An investigation of the influence of periodate on the proteolytic activity in extracts (incubation at pH 6.5) reveals that the activity has considerably increased at pH 7.7 (Table 9) as well as at pH 6.7 (Table 10). Both enzyme EI (pH 6.7) and enzyme EIII (pH 7.7) require intact SH groups for their function. Since periodate treatment of the egg material causes considerable decrease in activity of EI and EIII (Tables 16 and 17) it seems probable that the effect obtained after incubation must be attributed to an activation of enzyme EII. An inhibitor blocking enzyme EII may be destroyed by depolymerization. Some physiological observations support this concept.

It may here be of interest to discuss the effect of iodine. Iodine has an inactivating effect on EI, active at pH 6.7, as well as on enzyme EIII, active at pH 7.6 (Table 1). The SH groups belonging to enzymes EI and EIII are probably oxidized by iodine. It is not likely to influence possibly present inhibitors. This assumption is supported by the results in a previous paper (2) according to which the activity of the extract AX 20 (Table 5) containing EII is not influenced by iodine solution at pH 6.9 whereas it is increased by hydrogen peroxide which probably destroys the inhibitor. The occurrence of an inhibitor was already discussed in that paper.

The localization of the enzymes

The site of the proteolytic enzymes in the eggs has earlier been discussed. Several experiments indicate that enzyme EII is in some way attached to granules in the cortex and that these are altered by the influence of calcium. EII is then liberated simultaneously. Since the discovery of HEILBRUNN (30) that calcium causes a breakdown of plasma granules, the influence of calcium on such granules has been observed. According to most experiments enzyme EII, however, preferably occurs when the egg material has been washed in isotonic sodium chloride solution, i.e. when no calcium is added.

The fact that enzyme EI is easily obtained without splitting the granules might indicate that the enzyme occurs in a dissolved state in the cytoplasm. Other important observations contradict this assumption. Treatment (washing) in hypertonic solution which is assumed to dissolve granules gives a higher EI content (cf. Tables 20, 22–24 in (2)) than does treatment in sea water or isotonic saline solution.

Other experiments in the same paper (Table 25 (2)) revealed that enzyme EI accompanies granular particles to some degree. *Arbacia* eggs washed in isotonic sodium chloride solution were homogenized in that medium and centrifuged. In the opalescent supernatant low activity (17 mμA/ml) was obtained at pH 6.6, increasing 7 times with cysteine (EI) and three times with calcium. The sediment was extracted in distilled water and centrifuged. The supernatant was this time optically clear and was inactive at pH 6.6, but through addition of cysteine the activity reached 190 mμA/ml. Calcium had no effect. This proves that at least part of EI accompanies the particles and that the effect exerted by calcium is caused by its influence on the particles and not on the dissolved, though inactive enzyme EI. Thus from the beginning at least part of EI seems to have been somehow attached to the particles, although the homogenization procedure in itself is sufficient to liberate a greater or smaller part of the enzyme. HOLTER (31) pointed out this phenomenon in his investigations of proteinase from *Chaos chaos* and showed that homogenization liberates the enzyme attached to particles *in vivo*, so that proteinase after that process occurs in the ground cytoplasm. Regarding the localization in the sea urchin egg of enzyme EIII nothing can as yet be postulated.

The dissolving effect of calcium on cytoplasm granules in sea urchin eggs was thoroughly investigated by HULTIN (11) and later by GROSS (32) and by GOLDSTEIN on eggs of *Chaetopterus* (33). Apart from calcium the enzymes trypsin and papain (11) are also able to change the plasma granules (cf. DIANZANI (34)). ZOLLINGER (35) found that ribonuclease breaks down mitochondria which caused him to assume a high content of ribonucleic acids in mitochondria. LINDBERG and ERNSTER (36), however, pointed out that as the ribonucleic acid content in the mitochondria is evidently very low, the ribonucleic acids must be assumed to serve as a cement substance in these particles. These concepts are of interest also in this author's studies on ribonuclease (cf. (18, 19)).

Since it was proved that enzyme systems are activated upon fertilization, artificial parthenogenesis has become of interest also to chemists. The attention will here be called to physiological works by LILLIE, LOEB, HEILBRUNN, RUNNSTRÖM and others. The parthenogenis was usually brought about by treating the eggs with butyric acid and subsequent hypertonic treatment. It was believed that an incipient cytolysis of the egg surface was caused by the acid and that the subsequent hypertonic treatment stopped cytolysis. The influence of the medium on processes in the egg have been treated in this paper with special regard to the saline content and fertilization inhibiting or -promoting substances like periodate, clupein, *Fucus* factor etc.

As to calcium LINDAHL showed that absence of this substance prolonged the life time of the egg (37) and SCHECHTER (38) demonstrated that a decrease of the calcium content in sea water to $^1/_{12}$ and a simultaneous change of the pH value from 8.3 to 7.4 resulted in an about three times longer life time for 50 per cent of the eggs *(Arbacia punctulata)*. Calcium may primarily have a liberating effect upon structurally attached enzymes in the eggs, but might also activate enzymes, though also the contrary is possible. Thus e.g. NIHOUL (39) found that calcium inactivates a proteinase from *Vibrio cholera*.

It has been shown by RUNNSTRÖM, MONNÉ, and BROMAN (40) and later by KRAUSS (41) that upon fertilization in presence of cysteine and still more of thioglycolic acid at pH 7.8–8.0 the membrane elevation is facilitated, although its hardening is delayed. After some time the membrane is dissolved. The formation of —S—S— bonds which is considered to render the membrane tough and resistant fails to occur, thus apparently exposing it to the enzymes in the eggs.

Considering the scheme presented in the present paper it may seem paradoxical that membrane elevation occurs upon influence of thiol compounds, but the following might serve as an explanation. When activated by cysteine or thioglycolic acid EI may liberate EII or initiate some process which normally occurs in connection with EII. Membrane elevation is then brought about. As *SH groups* are already present in the beginning of the processes, the *—S—S— bonds* necessary for the resistance of the membrane cannot be formed. The membrane then begins to dissolve.

Summary

1. In extracts from the sea urchin species *Paracentrotus lividus, Arbacia lixula,* and *Echinocardium cordatum* the proteolytic activity has been determined viscosimetrically with gelatin as substrate.

2. Besides the previously found thiol enzyme EI (activity optimum at pH 6.7) and EII (activity around pH 7, inactivated by cysteine) another thiol enzyme called EIII (optimum at pH 7.6–7.8) has been found in the egg extracts.

3. Enzymatic differences occurred in the extracts of eggs from *Paracentrotus* which were washed in hypertonic sea water and homogenized either in distilled water or in 1 M KCl. In the KCl extract enzyme EII was predominant, whereas EI prevailed in the distilled water extract.

4. Heparin as well as cysteine considerably lowers the activity of enzyme EII.

5. Alloxan (fresh solutions) activates and stabilizes enzyme EII (measured at pH 7.7) as well as enzyme EIII. Enzyme EI is, however, inactivated by alloxan.

6. At pH 6.7 and 7.7 the activity in extracts of 8–12 minutes stages of fertilized eggs of *Paracentrotus* and *Echinocardium* was stronger than in the unfertilized eggs, i.e. EI as well as EIII were activated. The activity value of EII was low and did not increase upon fertilization.

7. Unfertilized *Arbacia* and *Paracentrotus* eggs which were treated with sodium periodate (3×10^{-5} M) and thereafter homogenized, exhibited lower acticity of EI and EIII. Physiological experiments revealed that fertilization was considerably facilitated upon sodium periodate treatment which seemed to increase the proportion of EII.

8. A substance prepared from *Fucus vesiculosus* (E. VASSEUR) prevented fertilization and seemed in these experiments to inactivate EII and EIII. This substance did not influence enzyme EI.

9. Jelly coat preparations from *Echinus esculentus* contained EII which was activated with periodate and iodoacetamide. As solutions of cautiously removed jelly coats were lacking in proteolytic activity, this activity may be attributed to enzymes leaking out from the egg.

10. Proteolytic activity in extracts from hatching blastula of *Paracentrotus* was demonstrated at pH 8.9. The enzyme did not occur in extracts from unfertilized eggs.

11. A survey of the role of the proteolytic enzymes in the egg before and after fertilization has been outlined.

ACKNOWLEDGEMENTS

The author wishes to thank Professor K. MYRBÄCK for his interest and for valuable suggestions and Professor J. RUNNSTRÖM for encouragement and support.

Thanks are also due to the Director af the Zoological Station at Naples, Professor R. DOHRN, to the Director of Laboratoire Arago at Banyuls-sur-Mer, Professor G. PETIT, and to Professor N. HOLMGREN and Dr. G. GUSTAFSON, the Zoological Station, Kristineberg, for laboratory facilities and material.

The author further wishes to thank Miss S. MILLER and Eng. I. LUNDBLAD for technical assistance.

The investigations were financially supported by grants from *Statens Medicinska Forskningsråd* and *Riksföreningen för kräftsjukdomarnas bekämpande* and by scholarships from *Stiftelsen Lars Hiertas Minne* and *Hierta-Retzius' Stipendiefond*, which is hereby gratefully acknowledged.

From Wenner-Grens Institut för Experimentell Biologi, Stockholms Högskola, Stockholm.

REFERENCES

1. Lundblad, G., Exptl. Cell Research *1,* 264 (1950).
2. Lundblad, G., Arkiv Kemi *4,* 537 (1952).
3. Lundblad, G., and Lundblad, I., Arkiv Kemi *6,* 387 (1953).
4. Ishida, J., Annotationes Zoologicae Japonenses *15,* 453 (1935–6).
5. Kopac, M. J., J. Cellular Comp. Physiol. *18,* 215 (1941).
6. Sugawara, H., J. Faculty Sci. Univ. Tokyo Sect. IV., *6,* 109 (1943).
7. Hultin, E., Svensk Kem. Tidskr. *60,* 40 (1948).
8. Harding, C. V., and Harding, D., Arkiv Zool. *3,* 357 (1952).
9. Lundblad, G., Arkiv Kemi *7,* 169 (1954).
10. Wicklund, E., Arkiv Zool. 1954. In press.
11. Hultin, T., Exptl. Cell Research *1,* 159 (1950). Ibid. *1,* 272 (1950).
12. Runnström, J., Biol. Bull. *69,* 351 (1935).
13. Kriszat, G., and Runnström, J., Exptl. Cell Research *3,* 500 (1952).
14. Runnström, J., and Kriszat, G., Arkiv Zool. *4,* 165 (1952).
15. Runnström, J., Arkiv Zool. *40A:* No. 17 (1948).
16. Endo, Y., Exptl. Cell Research *3,* 406 (1952).
17. Wicklund, E., Arkiv Zool. *40A:* No. 5, 1–18 (1947).
18. Lundblad, G., Arkiv Kemi *7,* 159 (1954).
19. Lundblad, G., and Hultin, E., Exptl. Cell Research *6,* 249 (1954).
20. Harding, C. V., Exptl. Cell Research *2,* 403 (1951).
21. Roth, J. S., Nature *171,* 127 (1953).
22. Lindvall, S., and Carsjö, A., Arkiv Kemi 1954. In press.
23. Irwing, G. W., Fruton, J. S., and Bergmann, M., Biol. Chem. *144,* 161 (1942).
24. Hanson, H. T., and Smith, E. L., J. Biol. Chem. *179,* 789 (1949).
25. Herberts, G., Upsala Läkareförenings Förhandl. *LVIII,* 253 (1953).
26. Siliprandi, N., and Daghetta, A., Boll. soc. ital. biol. sper. *26,* 1623 (1950).
27. Siliprandi, N., and Sommariva, O., Boll. soc. ital. biol. sper. *26,* 1625 (1950).
28. Talafant, E., and Sevela, M., Chem. Listy *46,* 182 (1952).
29. Patterson, J. W., Lazarow, A., and Levey, S., J. Biol. Chem. *177,* 197 (1949).
30. Heilbrunn, L. V., An outline of general physiology. Philadelphia 1945.
31. Holter, H., and Løvtrup, S., Compt. rend. trav. lab. Carlsberg. Sér. Chim. *27* nr 2, 27 (1949).
32. Gross, P. R., Biol. Bull. *105,* 365 (1953).
33. Goldstein, L., Biol. Bull. *105,* 87 (1953).
34. Dianzani, M. U., Experentia *9,* 343 (1953).
35. Zollinger, H. U., Revue d'Hématologie *5,* 696 (1950).
36. Lindberg, O., and Ernster, L., Protoplasmatologia. Springer-Verlag, Wien 1954.
37. Lindahl, P. E., Acta Zoologica *17,* 179 (1936).
38. Schechter, V., Biol. Bull. *72,* 366 (1937).
39. Nihoul, E., Compt. rend. soc. biol. *146,* 1394 (1952).
40. Runnström, J., Monné, L., and Broman, L., Arkiv Zool. *35A:* No. 3, 1–32 (1943).
41. Krauss, M., J. Exptl. Zool. *114,* 279 (1950).

Tryckt den 12 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 18

Communicated 10 March 1954 by JOHN RUNNSTRÖM and KARL MYRBÄCK

Proteolytic activity in sea urchin gametes

V. The influence of ribonuclease upon the proteolytic activity of egg extracts

By GUNNAR LUNDBLAD

Studies of the proteolytic enzymes in sea urchin eggs (1, 2, 3, 4) revealed that these enzymes were often difficult to extract from homogenized or lyophilized egg material. As it turned out that one or more of these proteolytic enzymes are, at least to a certain extent, associated with particles in the cytoplasm, and as it is known that the mitochondria contain nucleoproteins (5, 6), it seemed probable that proteolytic enzyme(s) is (are) bound to nucleoproteins. On the basis of MYRBÄCK's (7, 8) work (cf. Discussion) some enzymes have been tested as a means of liberating proteolytic enzymes in sea urchin eggs. Preliminary results obtained with ribonuclease have recently been communicated (9).

The present work gives a more detailed report on similar experiments and on the conditions under which they were carried out.

The activity of the following proteolytic enzymes has been estimated in extracts from unfertilized eggs:

Enzyme	pH optium	Influence of cysteine
Cathepsin II	4.3	Activating
E I	6.7	,,
E II	Around 7	Inactivating
E III	7.8	Activating

According to a scheme outlined by the author (4) EII is inactivated when EI and EIII become active. Very likely these enzymes are bound to particles, although a homogenization procedure often seems sufficient to liberate the enzymes to a varying extent from their granular association.

Material and methods

The investigated material consisted of eggs from the sea urchin species *Echinocardium cordatum* (frozen eggs), *Echinus esculentus* (frozen eggs), *Brissopsis lyrifera* (frozen ovaries with oocytes), and *Arbacia lixula* (lyophilized eggs). The experiments were carried out during autumn 1953 in Stockholm.

The proteolytic activity was determined viscosimetrically with gelatin as substrate and calculated on basis of a formula by HULTIN[1] (10).

The measurements were carried out in Ostwald viscosimeters and the reaction mixture consisted of 1.00 ml extract and 3.00 ml 4.00 per cent gelatin, buffered in acetate, imidazol or veronal. In some experiments the extract was first incubated with 0.50 ml cysteine or water. All cysteine solutions were 0.01 M and the pH was adjusted to 6.5. A crystalline ribonuclease preparation from Worthington lab. was used throughout the experiments. The gelatin solutions were sterilized with merthiolate, 0.005 per cent. For further details see previous papers (1–4).

The experiments reported in the previously mentioned paper (9) were carried out on lyophilized eggs of *Paracentrotus*. In presence of ribonuclease a higher proteolytic activity was obtained. In order to get the best effect of the added ribonuclease solution it was necessary to make the extraction run for a longer time period, i.e. 10–12 hours. The extractions were made in the cold. Owing to insufficient supply of *Paracentrotus* material, experiments were continued on material from other species, the egg material of which was not lyophilized. Only from *Arbacia* lyophilized egg material was available.

Results

Extraction in the cold

Eggs of *Echinocardium*, frozen and stored at — 18° C for $1\,^{1}/_{2}$ months, were used. The preparation of the eggs was made the following way. Fresh unfertilized *Echinocardium* eggs were washed three times in sea water, the eggs were suspended in dist. water and frozen. The thawed egg mash (pH 7.1) was divided in three equal parts, each 15 ml. To part I, 3 ml distilled water was added; to part II, 3 ml 0.03 per cent bee venom solution (lecithinase); and to part III, 3 ml 0.03 per cent ribonuclease solution. The suspensions were shaken for 18 hours in the cold and then centrifuged for two hours at 5 000 g.

Before measuring the proteolytic activity 1.00 ml extract was incubated with 0.50 ml distilled water or 0.01 M cysteine solution for 20 minutes at 20° C, whereafter 3 00 ml 4.00 per cent gelatin was added and the measurement started. The activity at pH 6.7 was stronger in the distilled water extract (68 mμA/ml) than in the ribonuclease extract (39 mμA/ml) without addition of cysteine, and very low in both extracts upon addition of cysteine (3 mμA/ml). After addition of cysteine the activity at pH 7.7 was stronger in the ribonuclease extract (90 mμA/ml) than in the dist. water extract (23 mμA/ml). When no cysteine was added the activity at pH 7.7 was 28 and 11 mμA/ml respectively. The results (cf. Discussion) indicate that enzyme EII is obtained in greater quantities upon extraction *without* ribonuclease, but that enzyme EIII is preferably obtained upon extraction *with* ribonuclease. The extraction with lecithinase gave the same activity values as the dist. water extraction.

---

[1] HULTIN's formula $A^{t^\circ} = J \cdot c^2{}_s \cdot \dfrac{d\dfrac{1}{\eta_{\mathrm{sp}}}}{dt}$

where A = the enzymic activity at t° C (units per gram) in the reaction mixture,
J = a factor for the influence of e.g. pH (for most substrates $J = 1$; independent of pH),
c_{s} = the concentration of the substrate in the reaction mixture (gram substrate per gram solution),
η_{sp} = specific viscosity, and
t = time in seconds.

In the continued experiments egg material from *Echinus esculentus* was used. The eggs were deprived of their jelly coats and frozen in a small amount of sea water. Part of the frozen material was suspended in distilled water to a suitable consistency. The suspension was treated in a glass homogenizer and divided in two parts: (A) 8.0 ml egg suspension + 2.0 ml distilled water; (B) 8.0 ml egg suspension + 2.0 ml 0.03 per cent ribonuclease solution. The suspensions were kept in the cold for 16 hours (pH 6.5) and centrifuged for 10 min. at 5 000 g. Nitrogen content: (A) 2.6 and (B) 2.8 mg/ml. Before measuring 1.00 ml extract was incubated with 0.50 ml distilled water or cysteine solution for 20 minutes at 20° C and then mixed with 3.00 ml gelatin. The activity was measured at pH 6.7 and 7.6 and also at pH 4.5. The proteolytic activity was then found to be particularly strong (520 mμA/ml) at pH 4.5 (cathepsin II type), but equal in both extracts *with*, as well as *without* cysteine. At pH 6.7 the activity was higher without cysteine in extract (A) (43 mμA/ml) than in (B) (9 mμA/ml) but lower with cysteine in (A) (53 mμA/ml) than in (B) (88 mμA/ml). At pH 7.6 the activity was higher in (B) than in (A) without cysteine (20 and 7 mμA/ml resp.), as well as with cysteine (65 and 20 mμA/ml resp.). Ribonuclease thus favoured the dissolution of EI, as well as of EIII. Similar investigations with crystalline desoxyribonuclease, Worthington Lab., revealed that this enzyme had no influence on the liberation of the proteolytic enzymes.

Since ribonuclease has the character of a basic protein (the isoelectric point (IEP) is between pH 7.8 and 9.5, dependent upon the ionic strength of the medium), some extractions were also carried out in the presence of clupein (IEP around pH 12).

Egg material from *Echinus* was used. Five ml frozen egg suspension was thawed in 10 ml distilled water. The mixture was homogenized in a glass tube and divided in three equal parts: (A) 5.0 ml egg suspension + 5.0 ml distilled water; (B) 5.0 ml egg suspension + 5.0 ml clupein (Hoffmann La Roche) 0.03 per cent; and (C) 5.0 egg suspension + 5.0 ml ribonuclease 0.03 per cent. The suspensions were kept at room temperature for 15 minutes, thereafter at + 5° C for 18 hours and then centrifuged. One of the experimental series is shown in Table 1. From this is evident that clupein has no effect on the proteolytic activity at pH 6.6. Ribonuclease, on the other hand, doubles its activity in presence of cysteine. At pH 7.7 the activity is very high in the clupein extract during the first half hour and still higher in the ribonuclease extract (C), compared to the control extract (A). The capacity of ribonuclease to liberate proteolytic enzymes at pH 7.7 (EIII) may partly be ascribed to its quality as basic protein. The basic protein effect of clupein must, however, be considered greater than that of ribonuclease, due to its IEP (pH 12). Therefore, only a small part of the capacity of this enzyme to liberate proteolytic enzymes may be ascribed to its non-enzymatic activity.

The author found that extracts from oocytes, which in the sea urchin material available can only be studied in *Brissopsis lyrifera*, exhibit very strong catheptic activity, but none or very insignificant proteolytic activity around neutral pH.

Table 1. Proteolytic activity in extracts from *Echinus* eggs treated with clupein or ribonuclease during the extraction.

1.00 ml extract was incubated for 20 minutes at 20° with 0.50 ml cysteine, mixed with 3.00 ml gelatin and measured.

Expt. No.	Treatment at extraction	mg N/ml	pH	mμA/ml	mμA/mg N
1	H_2O	2.0	6.6	32	16
2	Clupein	2.0	6.6	32	16
3	Ribonuclease	2.1	6.6	60	29
4	H_2O	2.0	7.7	15	7.5
5	Clupein	2.0	7.7	130	65
6	Ribonuclease	2.1	7.7	220	105

It seemed reasonable to assume that the neutral enzymes in the oocytes occur in an inactive form, e.g. bound to nucleoproteins, and that they can therefore be dissolved through influence of ribonuclease. In Table 2 the results of a series of measurements of an extract from *Brissopsis* are presented. Ovaries washed in sea water containing oocytes were frozen, kept at — 18° C and thawed immediately before extraction. To the thawed material 7 ml distilled water was added. The material was cooled and homogenized in a tissue grinder (GEKA, Tübingen) for three minutes. The obtained suspension (17 ml) was divided in two parts:

(A) 8.0 ml suspension + 2.0 ml distilled water, (B) 8.0 ml suspension + 2.0 ml 0.03 per cent ribonuclease solution. The preparations were frozen in a refrigerator, thawed and kept in the cold for 12 hours and then centrifuged. Nitrogen content: (A) 1.00, (B) 0.98 mg/ml.

As is evident from Table 2, the activity at pH 6.7 in the extract not treated with ribonuclease is very low, although it increases somewhat after addition of cysteine. When no cysteine is added the proteolytic activity in the ribonuclease-treated extract is also very low, but when adding cysteine very strong activa-

Table 2. Proteolytic activity in extracts from *Brissopsis* ovaries and oocytes treated with ribonuclease during extraction.

1.00 ml extract was incubated for 20 minutes at 20°C with 0.50 ml reagent, mixed with 3.00 ml gelatin and measured.

Expt. No.	Extraction	Reagent	pH	mμA/ml	mμA/mg N
7	H_2O	H_2O	6.7	3.0	3.0
8	H_2O	Cysteine	6.7	7.4	7.4
9	Ribonuclease	H_2O	6.7	7.8	8.0
10	,,	Cysteine	6.7	520	530
11	H_2O	H_2O	7.7	6.4	6.4
12	H_2O	Cysteine	7.7	14	14
13	Ribonuclease	H_2O	7.7	9.6	9.8
14	,,	Cysteine	7.7	27	28

tion sets in. At pH 7.7 the activity is stronger in the ribonuclease extract than in the dist. water extract. Cysteine enhances the activity further. Ribonuclease thus promotes the liberation of enzymes EI and EIII. The catheptic activity measured at pH 4.5 was as usual very strong (550 mμ*A*/ml). It was equally strong in both extracts, and cysteine exerted no influence.

Extracts from lyophilized *Arbacia* eggs were also investigated. The proteolytic activity was, however, low (the material was stored for 2 $^1/_2$ years) and never exceeded 10 mμ*A*/ml.

Experiments with ribonuclease added immediately before measuring the proteolytic activity in extracts of *Echinus* and *Brissopsis*

Previous experiments reveal that the proteolytic activity increases in presence of ribonuclease during extraction. The question then arises whether ribonuclease acts on the undissolved material or on substances dissolved in the extract. If the latter is the case, addition of ribonuclease to the extract immediately prior to viscosimetrical measuring ought to result in increased activity. This did not prove to be the case for extracts from frozen eggs of *Echinus,* nor for extracts of frozen *Brissopsis* ovaries and oocytes. This indicates that the action of ribonuclease concerns undissolved material.

No proteolytic activity of the ribonuclease itself was found when tested in the presence or absence of cysteine at pH 6.6 and 7.7 with gelatin as substrate.

Extraction at higher temperature

Experiments were also carried out extracting egg material at higher temperatures (25 and 30° C) during different time periods in presence of ribonuclease. Both *Echinocardium* and *Echinus* eggs were used in these experiments.

Echinocardium eggs frozen in distilled water were thawed and the material was divided in two parts: (A) 5.0 ml egg suspension + 2.0 ml distilled water, and (B) 5.0 ml egg suspension + 2.0 ml 0.03 per cent ribonuclease solution. The suspensions were rubbed in a glass homogenizer and samples were taken and kept for 15 min. and one hour, respectively, at 25° C (pH 7.2). They were then cooled down and centrifuged. 1.00 ml centrifuged extract was incubated with 0.50 ml cysteine at 20° C for 10 minutes. It was then mixed with 3.00 ml gelatin and measured at pH 6.7. The activity was about 60 mμ*A*/ml in both extracts after 15 minutes, but after one hour's incubation at 25° C the activity in the control was 82 mμ*A*/ml, and 120 mμ*A*/ml in the ribonuclease sample. Increased activity in presence of cysteine indicates liberation of enzyme EI.

Experiments on extracts from *Echinus,* the results of which are recorded in Table 3, were carried out as follows. Twenty ml frozen *Echinus* eggs were thawed in 40 ml distilled water, the suspension was treated in a glass homogenizer and divided in two parts: (A) 30.0 ml egg suspension + 10.0 ml distilled water, (B) 30.0 ml egg suspension + 10.0 ml 0.03 per cent ribonuclease. These mixtures were kept for two hours at + 5° C. Double samples of 3.0 ml each were taken and heated for different time periods at 30° C, and were then cooled and centrifuged.

Table 3. Proteolytic activity in extracts of *Echinus* eggs treated with ribonuclease during extraction at +30°C.

1.00 ml extract was incubated for 15 minutes at 20°C, mixed with 3.00 ml gelatin and the activity measured at pH 6.7.

Expt. No.	Extraction	Time of incubation at 30°C, in min.	mμA/ml
15	H_2O	0	25
16	Ribonuclease	0	26
17	H_2O	15	25
18	Ribonuclease	15	58
19	H_2O	30	35
20	Ribonuclease	30	66
21	H_2O	60	34
22	Ribonuclease	60	36
23	H_2O	120	42
24	Ribonuclease	120	26
25	H_2O	180	8
26	Ribonuclease	180	2

It is evident from Table 3 that the proteolytic activity, which was originally the same in all extracts, increased more than 50 per cent in the ribonuclease sample already after 15 minutes at 30° C and still more after 30 minutes. After one hour the activity decreases again and continues to decline in the two and three hours samples. In the control samples the activity increased somewhat after 30 minutes incubation and reached its highest value after two hours. The experiment indicates that the speed of the enzyme liberation is different in the control extract and in the ribonuclease extract. The former extracts only reaches $^2/_3$ of the highest value of the ribonuclease extract. The experiments also indicate that the liberation of enzyme EI is more rapidly brought about in the ribonuclease samples, where also higher activity values are obtained. The rapid decrease of the activity seems to be due to the inactivating effect of the dissolved enzyme EII and the temperature.

Discussion

A necessary condition for enzymatic investigations is to obtain enzyme activities sufficiently strong to make accurate measurements possible within a reasonable time period. One of the main difficulties in this series of investigations was to obtain extracts with sufficiently strong proteolytic activity. In most preparations inactive extracts and extracts not possible to activate with cysteine were obtained. Even when activity was observed it was often necessary to extend the activity measurements for a time period of 20 hours and more. This was especially the case when the eggs were difficult to fertilize or when the subsequent development was not quite normal.

It is only natural that biological material of this type shows varying qualities. The total amount of free and blocked proteolytic enzymes may thus change and the relation between the amount of free and blocked enzyme may vary. Such variations may be due, among other things, to the maturity of the eggs and seasonal environmental conditions. By varying the extraction conditions the present writer tried to gain as much as possible of the total enzyme amount. Such conditions were e.g. different pretreatments of the eggs, different extraction media, and varied technique during extraction.

The author further assumed that the enzyme might be blocked by association with structural cell components as shown by MYRBÄCK for β-amylase. He demonstrated (7, 8) that β-amylase of barley, partly bound to insoluble proteins and liberated upon germination, could also be liberated from ungerminated barley by means of proteolytic enzymes, such as papain.

It may be assumed that the proteolytic egg enzymes are blocked by proteins (or other substances), e.g. inhibitors with peptide linkages which are not split *in vitro* by proteolytic egg enzymes, but by other proteolytic enzymes, e.g. papain. Papain was also tested by the present writer (2), but since it became activated itself, its possible liberation of proteolytic egg enzymes could not be estimated. Therefore papain could not be used for this purpose. Papain could, however, be utilized as a means of testing the effect of different treatments of the egg material.

As a continuation of these investigations the experiments with ribonuclease were carried out. Far greater amounts of EI and EIII were extracted in presence than in absence of ribonuclease. The opposite conditions apparently prevailed regarding EII. As previously mentioned, this enzyme loses its activity in crude extracts upon addition of cysteine. Considering the present experimental results as well as those obtained previously, the following explanation of this contradictory behaviour seems to be most probable.

In the extracts where EII activity was observed the amount of EII was considerably greater than that of EI and EIII. Enzymes EI and EIII are very likely able to break down EII. The amount of EI and EIII was considerably increased upon extraction in presence of ribonuclease. The amount of EII is very likely increased during the first phase of the extraction procedure. However, since EI and EIII act upon EII, the increase in EI and EIII causes a decrease of the total amount of EII. This becomes still more noticeable upon addition of cysteine. This reagent strongly increases the activity of EI and EIII. Even if cysteine has no actual influence on EII the amount of this enzyme is decreased in presence of cysteine, due to the strong activation of enzymes EI and EIII. Whether cysteine has any direct influence on EII can only be found after purification of this enzyme.

The results discussed here agree well with the scheme of the appearance of the proteolytic enzymes upon fertilization as outlined by this author in a previous paper (4). In that scheme it was assumed that the thiol enzymes EI and EIII become active by influence of enzyme EII which is activated upon fertilization. Then, by action of EI and EIII, EII loses its own activity.

An investigation of the influence of ribonuclease on the proteolytic enzymes in oocytes from *Brissopsis* compared with the enzymatic activity in sea urchin eggs will be briefly discussed below.

In the absence of ribonuclease the proteolytic activity of enzymes EI, EII and EIII was very low in all previous experiments. (It should be remembered, however, that the catheptic enzyme exhibits about the same strength in oocytes extracts as in egg extracts.) The proteolytic activity is strongly increased when mashed oocytes are extracted upon addition af ribonuclease. An activation of the same order of magnitude as in extracts (without addition of ribonuclease) from matured, easily fertilized eggs might be the result of this procedure, as has hitherto been stated for enzyme EI. This may be of considerable importance in the fertilization process.

It is particularly interesting in this connection to remember that ribonuclease has been found in the sea urchin egg by BERNSTEIN (11) and later studied by LINDVALL and CARSJÖ (12), although its physiological role was not further investigated. It seems possible that ribonuclease and the proteolytic enzyme systems together play an important role during the developmental phase from oocyte to a fully matured, easily fertilized egg.

Summary

The influence of ribonuclease on the proteolytic activity of extracts from sea urchin eggs has been studied. The activity was viscosimetrically determined with gelatin as substrate.

The extraction of the proteolytic enzymes with optimum activity at pH 6.7 (EI) and 7.7 (EIII) from a mash of *Echinus* eggs was enhanced by addition of ribonuclease.

In the *Echinocardium* material available, the extraction of the proteolytic enzyme EIII (pH 7.7) was considerably enhanced by addition of ribonuclease. In the same material, the proteolytic activity of a water extract measured at pH 6.7 was strongly inhibited by cysteine, which shows that enzyme EII was predominant over EI. The EII activity was considerably lower after extraction in presence of ribonuclease. This is in accordance with the theory that EII rapidly vanishes in presence of EI and EIII which are liberated by ribonuclease.

In extracts from *Brissopsis* ovaries and oocytes very strong increase of enzyme EI was noticed in ribonuclease treated extracts.

The proteolytic activity at pH 4.5 (cathepsin II type) was strong in all extracts and was not influenced by ribonuclease.

The effect of ribonuclease seems to occur through its action upon undissolved material.

Extraction at 25°–30° C, brings about higher activity of EI in ribonuclease extracts, but the activity decreases during prolonged extraction.

Desoxyribonuclease had no influence upon the proteolytic activity when tested in the same way as ribonuclease.

ACKNOWLEDGEMENTS

The author wishes to thank Professor K. MYRBÄCK and Professor J. RUNNSTRÖM for their interest and for valuable suggestions, and Dr. E. HULTIN for interesting discussions.

He also wishes to thank Miss S. MILLER for technical assistance.

The investigations were financially supported by a grant from *Statens Medicinska Forskningsråd* and by a scholarship from *Hierta-Retzius' Stipendiefond,* which is gratefully acknowledged.

Wenner-Grens Institut för Experimentell Biologi, Stockholms Högskola, Stockholm.

REFERENCES

1. LUNDBLAD, G., Exptl. Cell Research *1*, 264 (1950).
2. —— Arkiv Kemi *4*, 537 (1952).
3. LUNDBLAD, G., and LUNDBLAD I., Arkiv Kemi *6*, 387 (1953).
4. LUNDBLAD, G., Arkiv Kemi *7*, 127 (1954).
5. ZOLLINGER, H. U., Revue d'Hématologie *5*, 696 (1950).
6. LINDBERG, O., and ERNSTER, L., Protoplasmatologia, Wien 1954.
7. MYRBÄCK, K., and MYRBÄCK, S., Biochem. Z. *258*, 158 (1933); *285*, 282 (1936).
8. MYRBÄCK, K., and ÖRTENBLAD, B., Enzymologia *3*, 305 (1938); *7*, 176 (1939).
9. LUNDBLAD, G., and HULTIN, E., Exptl. Cell Research *6*, 249 (1954).
10. HULTIN, E., Svensk Kem. Tidskr. *60*, 40 (1948).
11. BERNSTEIN, H. M., Biol. Bull. *97*, 255 (1949).
12. LINDVALL, S., and CARSJÖ, A., Arkiv Kemi. In press.

Tryckt den 13 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 19

Communicated 10 March 1954 by JOHN RUNNSTRÖM and KARL MYRBÄCK

Proteolytic activity in sea urchin gametes

VI. A study of a proteolytic enzyme in extracts of spermatozoa

By GUNNAR LUNDBLAD

With 4 figures in the text

In two previous papers proteolytic activity in extracts of sea urchin eggs and spermatozoa was reported (1, 2). The proteolytic activity in extracts of sea urchin eggs has been further studied (3, 4, 5, 6, 7). When examining the hyaluronidase activity in extracts from sea urchin spermatozoa, some results concerning the proteolytic activity in such extracts were also recorded (8).

In the present study continued experiments were carried out on the proteolytic enzyme activity in extracts from fresh or lyophilized spermatozoa. Since the activity in sperm extracts from *Arbacia* is considerably stronger than in extracts from other species, and since the supply of *Arbacia* was more abundant, only this material was used in the present study. In this work proteolytic activity was found at two separate pH values, 3 and 6–10. However, only data from the activity occurring nearest to the physiological pH interval are given here.

Material and methods

Material from the sea urchin species *Arbacia lixula,* collected in the Gulf of Naples, was used. The work was carried out at the Zoological Station of Naples during winter and spring 1949 and 1951 and in Stockholm in 1950. The sperm material was obtained by injecting 0.5 ml 0.5 potassium chloride in the male sea urchin, according to TYLER (9). The injected sea urchins were placed in Boveri discs containing sea water and the sperm was obtained in this medium. The sea water was removed from the sedimented sperm by suction and the material was then lyophilized. In experiments on the fresh material, however, the sperm was first suspended in a large amount of sea water and subsequently centrifuged at 7 000–8 000 g. This procedure was repeated once and the material was then extracted in different ways.

The proteolytic activity was either determined viscosimetrically or by means of formol titration. In both cases solutions of gelatin served (Fischer Sc. Co.) as substrate. In these solutions 0.005 per cent merthiolate (Lilly) was used as a sterilizing agent.

Viscosimetrical determination.—Ostwald viscosimeters were used in these measurements. The reaction mixture consisted of 1.00 ml extract and 3.00 ml 4.00 per cent gelatin, and when the effect of certain substances was investigated, 0.50 ml reagent was added to the reaction mixture. The temperature was constantly 35.5° C during the measurements. The gelatin solutions were buffered with citrate, imidizol or boric acid-soda, respectively (ionic strength 0.1). The viscosimetrical values were calculated on the basis of HULTIN's formula[1] (10) and expressed in $m\mu A/ml$ ($m\mu A = A \times 10^{-9}$).

Formol titration.—The measurements were carried out according to SØRENSEN (11) and NORTHROP (12). The reaction mixture consisted of 3.00 ml extract, 3.00 ml buffer solution and 6.00 ml of a 6.00 per cent non-buffered gelatin solution. Two samples of 2.00 ml each were taken immediately after mixing and 3.0 ml 17 per cent formaldehyde solution was added to the samples. At the end of the incubation another two samples of 2.00 ml were taken and mixed with 3.0 ml formol. After addition of two drops phenolphtaleine (1.0 per cent in ethanol) the samples were titrated with 0.01 M KOH.

The pH values were checked by a Beckman glass electrode pH meter, model G, and the nitrogen was determined according to Kjeldahl (modified).

Results

1. The influence of the preparation on the activity

Measurements of the proteolytic activity in extracts from sea urchin sperm (2) have previously been carried out on lyophilized material using distilled water or 0.1 M acetic acid as extraction media. The activity was then usually somewhat stronger in the distilled water extracts than in the acetic acid extracts when calculated per ml, but the opposite was true when calculated per mg nitrogen in the extract. There was no difficulty in obtaining active solutions in extracts of lyophilized material. Extraction of the proteolytic activity from fresh sperm was, however, far more difficult. The extracts were either very weak enzymatically or completely lacking in activity. It was therefore necessary to carry out some systematic experiments and Table 1 gives an example of such an experimental series. The experiments reveal that three factors are needed in order to obtain strong activity in extracts from fresh spermatozoa: 1) Distilled water as extraction medium, 2) freezing and thawing, and 3) long extraction time after the thawing. It is only when combining these three factors that the strong activity shown in experiment 3 is brought about.

The activity in extracts from fresh lyophilized sperm was also investigated. *Arbacia* sperm ejaculated in sea water after injection of potassium chloride was

---

[1] HULTIN's formula: $A^{t°} = J \cdot c^2_s \cdot \dfrac{d\dfrac{1}{\eta_{sp}}}{dt}$

where A = the enzymic activity at $t°$ C (units per gram) in the reaction mixture
J = a factor for the influence of e.g. pH (for most substrates $J = 1$; independent of pH),
c_s = the concentration of the substrate in the reaction mixture (gram substrate per gram solution),
η_{sp} = specific viscosity, and
t = time in seconds.

Table 1. Extracts from washed sperm.

Fresh *Arbacia* sperm (KCl inj.) was twice suspended in sea water, centrifuged and the sediment divided in 4 parts.

A: the material was suspended in distilled water, frozen, thawed and centrifuged. — B: the material was suspended in sea water, frozen, extracted 5 days and centrifuged. — C: the material was suspended in distilled water and then treated like B. — D: the material was suspended in distilled water, extracted for 5 days without freezing and thawing and centrifuged. All operations were carried out in the cold (2–3°).

1.00 ml extract was mixed with 3.00 ml gelatin, and measured at pH 6.7.

Expt. No.	Extract		Treatment at extraction			mμ*A*/ml	mμ*A*/mg N
	Prep.	mg N/ml	Medium	Freezing-thawing	Time		
1	A	0.85	H_2O	+	—	0	0
2	B	1.38	Sea w.	+	5 days	7.0	5.1
3	C	1.27	H_2O	+	,, ,,	120	95
4	D	0.48	,,	−	,, ,,	13.0	27

washed in sea water and centrifuged at about 5 000 g. The sediment was suspended in an *equal* volume of distilled water, frozen and lyophilized. The dried material was extracted in distilled water (2.5 per cent dry weight), frozen, thawed, kept in the cold for 20 hours and centrifuged. Nitrogen content = 0.85 mg/ml. The proteolytic activity at pH 6.6 was 390 mμ*A*/ml and 460 mμ*A*/mg N. The sperm medium obtained from the first centrifugation possessed no proteolytic activity, nor has any proteolytic activity ever been found in sperm media from other species.

Table 2 shows some extraction experiments on lyophilized material stored for one year. It is evident from these experiments that the same activity and the same nitrogen content is obtained, whether a weak (physiological) or a strong sodium chloride solution is used. One molar potassium chloride as extraction

Table 2. Different extraction media for lyophilized sperm.

1.00 g lyophilized *Arbacia* sperm was suspended in 20 ml of the given medium, frozen, thawed and kept in the cold for 10 hours, then centrifuged, dialysed against 0.9 % NaCl for 20 hours and centrifuged. During the dialysis precipitates were formed in extracts II and III. Reaction mixture: 1.00 ml extract and 3.00 ml gelatin. pH in mixture 6.7.

Expt. No.	Extract		Extraction medium	mμ*A*/ml	mμ*A*/mg N
	Prep.	mg N/ml			
5	I	0.31	NaCl, 0.9 %	31	100
6	II	0.30	,, , 3.6 %	32	110
7	III	0.28	KCl, M	43	150
8	IV	0.07	HAc, M/10	7.8	110
9	V	0.31	H_2O	25	81

medium (MIRSKY, Science *84*, 333 (1936)) caused stronger proteolytic activity. Since the nitrogen content was lower in this extract, the increased activity is especially evident when calculated on nitrogen. The activity in acetic acid extracts is low when calculated on volume, but comparatively strong when calculated on nitrogen.

As proteolytic activity occurs in intestines and excrements, this material was extracted and then investigated according to the present technique. The intestinal enzyme is not influenced by alloxan as is the sperm enzyme.

2. Influence of some reagents upon the proteolytic activity of sperm extracts

In order to gain knowledge about the enzyme active at neutral pH, sperm extracts were treated with different substances. Cysteine did not exert any influence on the activity, neither alone nor when combined with other reagents. Certain oxidazing agents and some other reagents, particularly alloxan, have a certain inactivating effect. Heparin was shown by HARDING and HARDING (13, 14) to have a strong fertilization-inhibiting effect. Table 3 shows the effect of heparin and alloxan (fresh solution) on the proteolytic activity in an extract from fresh spermatozoa. It is evident that heparin has no influence on the activity. Yet it has previously been shown to inhibit one of the proteolytic egg enzymes, EII (cf. (5)). Alloxan decreases the activity about 30–40 per cent for both dialysed and undialysed extracts. During dialysis there was a precipitation of protein. As shown in experiments 12 and 14, 80 per cent of the activity disappeared during dialysis when calculated per volume, but only 30 per cent if calculated per nitrogen in solution.

Considering the strong inhibiting effect of alloxan on the sperm enzyme, some fertilization experiments were carried out in the presence of alloxan. *Arbacia* eggs were suspended in sea water containing 0.05 M alloxan. Only 5 per cent of the eggs were fertilized, but 95 per cent in the alloxan free control. As later

Table 3. Influence of heparin and alloxan upon extracts from washed sperm.

Extract I: Fresh *Arbacia* sperm (KCl inj.) was twice suspended in sea water, centrifuged, suspended in distilled water, frozen, thawed, extracted for 5 days, and centrifuged.

Extract II: Another batch of fresh material treated as I.

Extract IID: Extract II dialysed against distilled water at +3° for 40 hours and subsequent centrifugation. 1.00 ml extract was incubated with 0.50 ml agent for 1 hour at 20°, pH in mixture 6.7. Heparin from Hynson, Westcott, and Dunning Co (HWD).

Expt. No.	Extract		Reagent	mμA/ml	mμA/mg N	Rel. activity
	Prep.	mg N / ml				
10	I	1.3	H_2O	120	93	—
11	,,	,,	Heparin HWD, 0.05 %	,,	,,	—
12	II	3.4	H_2O	92	27	1.00
13	,,	,,	Alloxan, 0.005 M	64	19	0.70
14	IID	0.89	H_2O	16	18	1.00
15	,,	,,	Alloxan, 0.005 M	9	10	0.57

Table 4. Influence of iodine and hydrogen peroxide upon extracts from lyophilized sperm.

Extract I: Lyophilized *Arbacia* sperm was suspended in distilled water, frozen, thawed and kept 12 hours in the cold, centrifuged, dialysed against 0.9 % NaCl for 23 hours, and centrifuged again.

Extract II: Lyophilized *Arbacia* sperm suspended in distilled water, frozen, thawed and kept 17 hours in the cold, centrifuged, dialyzed against distilled water for 45 hours, and centrifuged again.

All operations in the cold. Reaction mixture: 1.00 ml extract was incubated with 0.50 ml agent for 1 hour at 20° and then mixed with 3.00 ml gelatin.

Expt. No.	Extract		Reagent	pH	mμA/ml	mμA/ mg N	Rel. activity
	Prep.	mg N / ml					
16	I	0.29	H_2O	6.7	52	180	1.00
17	„	„	I_2–KI, 0.003 M	„	45	160	0.87
18	„	„	H_2O_2, 0.5 %	„	36	120	0.69
19	II	0.024	H_2O	6.5	18	750	1.00
20	„	„	I_2–KI, 0.003 M	„	17	710	0.95
21	„	„	H_2O_2, 0.5 %	„	14	580	0.78

reported by WICKLUND (15), the effect seems to be due to paralyzation of the spermatozoa.

Table 4 gives the effect of iodine and hydrogen peroxide on two extracts of lyophilized spermatozoa. Extract I is dialyzed against physiological saline solution and extract II against distilled water. The results reveal that iodine lowers the activity to about 10 per cent and hydrogen peroxide to 25–30 per cent. A strong evolution of gas sets in when hydrogen peroxide is added to the sperm extract. No gas was evolved in the boiled control. Lord ROTHSCHILD showed that sea urchin spermatozoa contain catalase (16).

In Table 5 the influence of some other substances is shown. The plasmin inhibitor prepared by the writer as described in a previous paper (17) has a very

Table 5. Activity experiments on *Arbacia* extract.

Lyophilized *Arbacia* sperm was extracted in distilled water, frozen, thawed, kept in the cold for 20 hours, centrifuged and dialysed against distilled water for 40 hours.

1.00 ml extract was incubated 1 hour at 20°C with 0.50 ml agent and then mixed with 3.00 ml gelatin at pH 6.7. N = 0.12 mg/ml.

Expt. No.	Reagent	mμA/ml	mμA/ml N	Rel. activity
22	H_2O	43	360	1.00
23	Plasmin inhibitor (PI)	10	83	0.23
24	Porphyrindin, 0.001 M	42	35	0.98
25	Heparin, 0.05 % HWD	44	370	1.02
26	*p*-chloro-mercuri-benzoic acid, 10^{-4} M	41	340	0.95

strong inactivating effect. The effect on the egg enzyme which cannot be activated with cysteine was, however, completely inhibitory (3). The other agents do not seem to cause any inhibitory effect. As communicated earlier (4), heparin has no effect on the proteolytic activity of the thiol enzyme EI (from egg), active at pH 6.7, but inhibits enzyme EII (5) which is active at pH 7 and inactivated by cysteine.

3. Some investigations of the proteolytic activity of sperm extracts

Fig. 1 shows an activity-pH-curve made on a sperm extract and determined by formol titration. Lyophilized *Arbacia* spermatozoa (stored for 1 $^1/_2$ years and used also in the subsequent experiments) were suspended in distilled water (2.5 per cent dry weight). The suspension was frozen, thawed and kept in the cold for 20 hours. It was then centrifuged and dialyzed for 42 hours against distilled water. Nitrogen content = 0.082 mg/ml. The samples were kept at 35.5° C and measured after 17 and 74 hours. According to Fig. 1 the activity reaches its maximum at pH 8.5–9.0. Values obtained during measurements in acid solution indicated the occurrence of another proteolytic enzyme active around pH 3. This branch of the curve, however, is not included in Fig. 1, since the values were scattered. In order to investigate the enzymatic activity in the acid region the following experiment was carried out.

Arbacia sperm (same preparation as previously) was suspended in dist. water, frozen, thawed and kept for five hours, then frozen and thawed again and kept for another 10 hours in the cold. After centrifugation it was dialyzed against distilled water for 20 hours. Nitrogen content = 0.069 mg/ml. The extract was divided in two parts, A and B.

The sample A was kept at pH 1.5 for 30 minutes at 50° C. Sample B was kept at pH 8 under same conditions. The pH values were thereafter restored to 6.5. Both samples were dialyzed against distilled water in separate vessels in the cold. The proteolytic activity was measured viscosimetrically at three different pH values, i.e. 3.1, 6.5 and 8.2. The activity in A was 64, 7.8 and 0 mμA/ml, and in B 0, 0 and 20 mμA/ml. The original activity was 44 mμA/ml at pH 6.6. These values reveal strong proteolytic activity at pH 3.1 indicating that this enzyme is resistant to incubation in strongly acid solution, but is inactive at pH 8. As might be expected, the neutral enzyme previously studied was inactive after incubation in acid solution measured at pH 3.1, but resistant to incubation at pH 8.

As the next phase of investigation attempts to extend the extraction of sperm were made. In the first experiment alkaline extraction was followed by acid extraction.

Extract I: Lyophilized *Arbacia* sperm was extracted in soda-bicarbonate buffer, pH 8.5, frozen, thawed and kept 5 hours in the cold. The extract was centrifuged and dialyzed for 20 hours against tap water and again centrifuged.

Extract II: The residue from extract I was suspended in acidified distilled water, pH 1.7 (HCl), and extracted for 20 hours with freezing and thawing. The extract was centrifuged and dialysed against tap water (5°) for 20 hours and again centrifuged.

1.00 ml extract was mixed with 1.00 ml buffer and 2.00 ml of a 6.00 per cent gelatin solution.

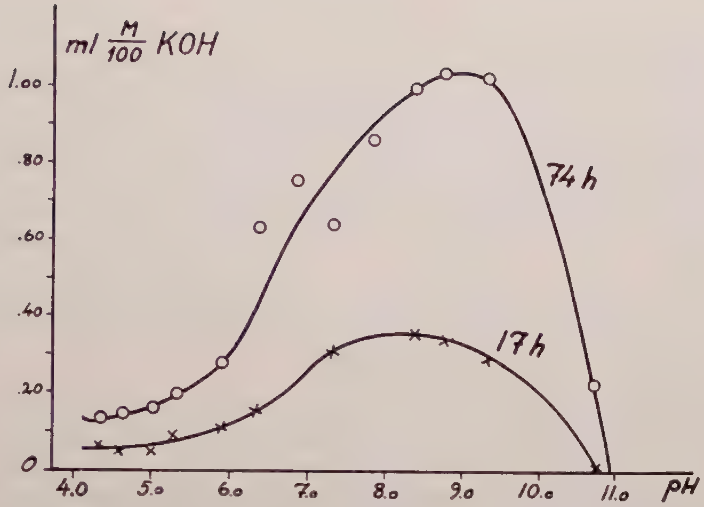


Fig. 1. Activity-pH-curve made on an extract from lyophilized *Arbacia* sperm (formol titration). The activity determined viscosimetrically at pH 6.6 = 40 mμ*A*/ml. N = 0.082 mg/ml.

The proteolytic activity was measured viscosimetrically at pH 3.8 (citrate), 6.6 (imidazol), and 8.4 (boric acid – soda). In extract I the values of the proteolytic activity were, in this order: 6.6, 64 and 98 mμ*A*/ml, and in extract II: 3.6, 4.8 and 4.8 mμ*A*/ml. Nitrogen content: Extract I = 0.11 mg/ml. Extract II = 0.15 mg/ml.

In the next series of experiments acid extraction followed by alkaline extraction was performed.

Extract I: Lyophilized *Arbacia* sperm was extracted in distilled water pH 1.9, acidified with HCl, for 15 hours with freezing and thawing. Centrifugation and dialysis against tap water (+ 5°) for 40 hours with subsequent centrifugation.

Extract II: The residue from extract I was suspended in soda-bicarbonate buffer pH 8.5 and extracted with freezing and thawing for 15 hours. Centrifugation and dialysis as extract I.

1.00 ml extract was mixed with 1.00 ml buffer and 2.00 ml of a 6.00 per cent gelatin solution.

The proteolytic activity at pH 3.8, 6.6 and 8.4 was, for extract I: 6.3, 1.8 and 2.4 mμ*A*/ml, and for extract II: 0.3, 2.7 and 5.4 mμ*A*/ml. Nitrogen content: Extract I = 0.064 mg/ml, extract II = 0.042 mg/ml.

From all these data it is evident that the alkaline extraction is useful but that the greatest part of the enzymes is destroyed during acid extraction. A pH value between 3 and 4 during extraction would be more suitable.

In Fig. 2 a stability-pH-curve is shown. The same material as earlier was extracted in distilled water (2.5 per cent dry weight) for 24 hours in the cold after freezing and thawing. After centrifugation it was dialysed against distilled water for 23 hours. Nitrogen content = 0.077 mg/ml. Each sample was incubated at a certain pH for 30 minutes at 40° C, the pH was corrected to

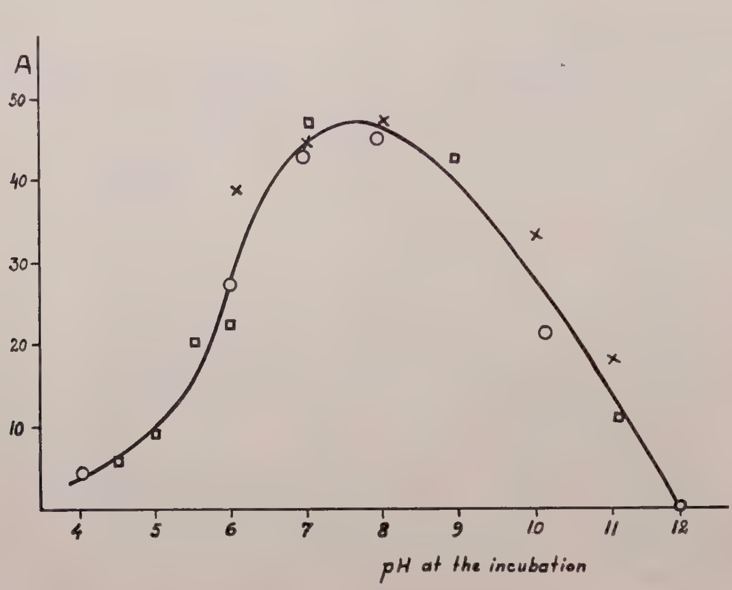


Fig. 2. Stability-pH-curve of the proteolytic enzyme which is active between pH 6 and 10.

The samples were treated for 30 minutes at 40°C at different pH values.
The activity was determined viscosimetrically at pH 6.5.

6.5, whereafter the activity was measured viscosimetrically. Corrections were made for change of volume at the different pH values. As seen from Fig. 2 the optimum stability is around pH 7.8, which is near the pH of the sea water. The stability pH of the acid enzyme could not be determined because of difficulties during measurements.

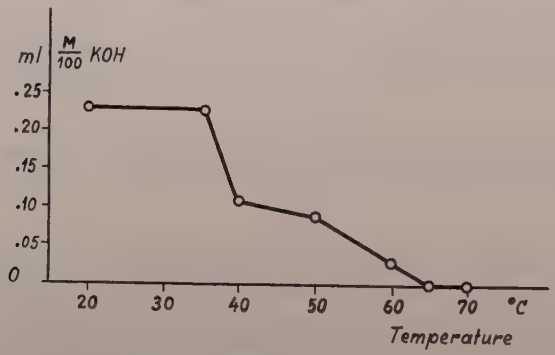


Fig. 3. Effect of temperature on the proteolytic enzyme which is active between pH 6 and 10.

The samples were kept for 30 minutes at different temperatures (pH 6.6) after which the proteolytic activity was estimated by formol titration. Calculated according to Arrhenius, the energy of activation is appr. $\mu = 15\,000$ cal/mol.

An investigation of the heat stability of the proteolytic activity in a sperm extract is shown in Fig. 3. The same extract (N = 0.069 mg/ml) was used as in an earlier series of experiments. The viscosimetrical activity was 44 mμA/ml. Samples were kept for 30 minutes at different temperatures at pH 6.6. The activity was measured by formol titration. Each sample was incubated with gelatin for 27 hours at 35.5° C and measured. The values in duplicate samples were in good agreement. Fig. 3 reveals that optimal activity is prevalent at 20 and 35.5° C but that the activity rapidly decreases when the temperature is increased, reaching zero at 65° C.

4. Some attempts to purify the proteolytic enzyme from sperms active at neutral pH

Several experiments were made in the attempt to purify the enzyme active around neutral pH. Fractionation with ammonium sulphate in the cold in a non-buffered solution revealed that the amount of precipitated enzyme was distributed within the various saturation intervals as follows: 0–35 per cent $AmSO_4$ sat. 13 mμA/ml (20 per cent of the total activity), 35–58 per cent sat. 4.8 mμA/ml (7.5 per cent), and 58–72 per cent sat. 2 mμA/ml (3 per cent). The activity was measured viscosimetrically at pH 7.1. The original activity was 65 mμA/ml. In order to make possible further purification of the extract, the isoelectric point (IEP) was determined by means of the TISELIUS apparatus (18) modified according to SVENSSON (19). Since the enzyme content in the extract was too low to cause optical activity, the mobilities were calculated by measuring the enzymatic activity in a divided cell (cf. DANIELSSON and SANDEGREN (20)). A series of measurements carried out on different extracts in-

Table 6. Activity experiment with purified *Arbacia* sperm extract.

Extract I: Lyophilized *Arbacia* sperm was extracted in distilled water for 20 hours with freezing and thawing. Centrifugation and dialysis against distilled water for 18 hours. All operations in the cold.

Extract II: Extract I adjusted to pH 4.6 and ammonium sulphate added to 25 per cent saturation. The solution was kept for 3 hours at zero, a precipitate was formed which was centrifuged off and dissolved in 0.9 per cent sodium chloride. The pH was adjusted to 6.8 (NaOH) and the solution dialysed against 0.9 per cent sodium chloride for 18 hours and then centrifuged. The supernatant is studied in the table. N = 0.07 mg/ml.

Reaction mixture: 1.00 ml extract is treated with 0.50 ml agent for 1 hour at 20°C and then mixed with 3.00 ml gelatin at pH 6.7 Each activity value is the average of 2 samples.

Expt. No.	Reagent	mμA/ml	mμA/mg N	Rel. activity
27	H_2O	37	530	1.00
28	$CaCl_2$, 0.1 M	38	540	1.03
29	Cysteine, 0.003 M	34	485	0.92
30	Cystine, 0.003 M	34	485	0.92
31	Alloxan, 0.005 M pH 6.5	10	140	0.27
32	CMB, 5×10^{-4} M	30	430	0.81
33	I_2–KI, 0.001 M	27	390	0.73
34	IBA, 5×10^{-4} M	29	410	0.78

dicated an IEP within the pH interval 4.4–4.6. The position of this pH value was confirmed by precipitation experiments. These revealed that the strongest proteolytic activity measured at pH 6.8 was obtained from the precipitate formed in this interval. At pH 4.6 the enzyme extract became strongly opalescent without addition of sulphate. When increasing or decreasing the pH value the extract again became transparent. In order to obtain a higher degree of purification ammonium $AmSO_4$ was added to an extract with pH adjusted to 4.6 (acetate buffer) up to different degrees of saturation. The precipitates were centrifuged down, extracted (dissolved) and dialyzed in 0.9 per cent NaCl at neutral pH (6.7–7.0), and measured viscosimetrically with regard to proteolytic activity. It was evident from these experiments that the greater part of the enzyme precipitates in the interval 0–25 per cent $AmSO_4$ sat. with most

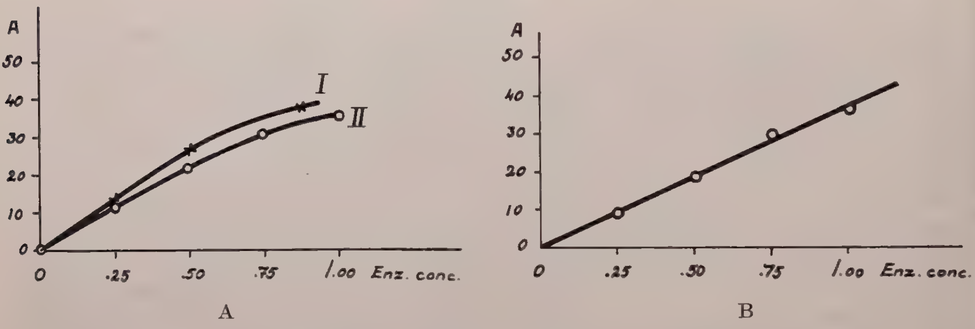


Fig. 4 A and B. Proteolytic activity as a function of enzyme concentration.

A: Crude extract, i.e. Extract I described in Table 6. I. Activity measured at pH 7.2. II. Activity measured at pH 6.7. — B: Purified extract, i.e. Extract II described in Table 6. The activity was measured at pH 6.7.

Reaction mixture: a ml extract + $(1.00 - a)$ ml distilled water were mixed with 3.00 ml gelatin and the activity determined viscosimetrically.

of it precipitating between 12 and 20 per cent. In all fractionation experiments a maximum of $^1/_3$ of the original enzyme activity was obtained. The proteolytic activity, calculated per nitrogen, was then seven times greater than the original activity.

In Table 6 some experiments on the activity of a purified enzyme are recorded. Fig. 4 shows the activity as a function of the enzyme concentration, both in the original and in the purified extract.

The experiments presented in Table 6 reveal that calcium ion has no influence on the proteolytic activity. Cysteine and cystine may have a weak inhibitory action. The SH inhibiting substances *p*-chloro-mercuribenzoic acid (CMB) and *o*-iodoso benzoic acid (IBA) lower the activity about 20 per cent. Alloxan has stronger inhibitory power in the purified extract than in the original extract (cf. Table 3).

Fig. 4 shows that a linear relationship between enzyme concentration and proteolytic activity has been reached by purification of the enzyme. In experiments on the original extract values diverging from the straight line indicate that the formation of the enzyme-substrate complex is disturbed in this extract.

Discussion

Viscosimetric measurements made on extracts of *Arbacia* sperm, reported in a former paper (2), gave an activity-pH-curve which had two optima, one at pH 4.2 and one at 6–7. The formol titration method used in the present experiments gave an activity-pH-curve which had two similar optima, one at pH 3–4 and one at pH 8–10. The effects of various substances upon the enzyme active at the latter pH optimum were studied and are discussed below.

Of the different substances tested, only two exhibited a strong inhibitory effect: alloxan and a plasmin inhibitor prepared from bovine serum. The inhibitory effect of alloxan may indicate that SH groups are needed for the function of the enzyme. On the other hand, alloxan is also known to react with chemically active groups other than the thiol group and, in the present experiments, cysteine proved to be ineffective either as an activating or a reactivating agent. The inactivating effect of both cysteine and cystine upon the purified enzyme is probably due to their amino acid configuration (HUNTER and DOWNS (21)).

Although the effect of alloxan on this enzyme may not involve SH groups, experiments concerning the effects of thiol reagents on living spermatozoa suggest that the *in vivo* effect of alloxan on spermatozoa may occur through its effect on SH groups. For example, alloxan promoted sperm paralysis (WICKLUND (15)); oxidized glutathion inhibited fertilization of *Pomatoceros* (MONROY (22)); certain substances known to react with SH groups also caused paralysis of spermatozoa, this effect being reversed to some extent by cysteine (MAC LEOD (23)). The content of glutathion is shown by BÄCKSTRÖM (24) to decrease during aging.

Summary

The proteolytic activity previously found in extracts from fresh or lyophilized sperm material of *Arbacia* has been further studied. The activity was measured viscosimetrically, as well as by formol titration using gelatin as substrate. The strongest activity in fresh material was found after several days' extraction in distilled water involving freezing and thawing. No proteolytic activity occurred in the sperm medium itself. Extraction of lyophilized material was less difficult.

Proteolytic activity was demonstrated at 2 different pH values, pH 3–4 and pH 6–10. The enzyme active at the latter pH value was studied in some detail. Its optimal stability occurs at pH 7.8 and it is stable at temperatures up to approx. 40° C (30 min. at pH 6.6). At 40° C the activity decreases rapidly; at 65° C it is inactive.

Cysteine exerts no activating effect on the proteolytic activity. Alloxan has a strong inhibitory effect which is probably due to its action on a functional group of the enzyme other than the SH group. Calcium ions and heparin do not affect the activity. A plasmin inhibitor prepared from blood serum has a strong inhibitory effect.

Experiments were carried out to purify the enzyme by ammonium sulphate fractionation at the isoelectric point (pH 4.4–4.6). Most of the enzyme pre-

cipitated below 25 per cent saturation. Cysteine and cystine inhibited slightly the proteolytic activity of the purified enzyme. A linear correlation exists between concentration of the purified enzyme and the proteolytic activity.

Acknowledgements

The author wishes to express his gratitude to Professor K. MYRBÄCK for the interest he has kindly taken in this work, and to Professor J. RUNNSTRÖM for encouragement and support.

Thanks are also due to the Director of the Zoological Station at Naples, Professor R. DOHRN.

The author further wishes to thank Eng. Å. STENHARDT for valuable technical assistance.

The investigations were financially supported by a grant from *Stiftelsen Lars Hiertas' Minnes Fond,* which is hereby gratefully acknowledged.

From Wenner-Grens Institut för Experimentell Biologi, Stockholms Högskola, Stockholm.

REFERENCES

1. LUNDBLAD, G., Nature *163,* 643 (1949).
2. LUNDBLAD, G., Exptl. Cell Research *1,* 264 (1950).
3. LUNDBLAD, G., Arkiv Kemi *4,* 537 (1952).
4. LUNDBLAD, G., and LUNDBLAD, I., Arkiv Kemi *6,* 387 (1953).
5. LUNDBLAD, G., Arkiv Kemi *7,* 127 (1954).
6. LUNDBLAD, G., and HULTIN, H., Exptl. Cell Research *6,* 249 (1954).
7. LUNDBLAD, G., Arkiv Kemi *7,* 159 (1954).
8. LUNDBLAD, G., and MONROY, A., Arkiv Kemi *2,* 343 (1950).
9. TYLER, A., Collecting Net *19,* 19 (1949).
10. HULTIN, H., Svensk Kem. Tidskr. *60,* 40 (1948).
11. SØRENSEN, S. P. L., Biochem. Z. *7,* 45 (1907).
12. NORTHROP, J. H., J. Gen. Physiol. *9,* 767 (1926).
13. HARDING, C. V., Exptl. Cell Research *2,* 403 (1951).
14. HARDING, C. V., and HARDING, D., Arkiv Zool. *3,* 357 (1952).
15. WICKLUND, E., Arkiv Zool. In press.
16. ROTHSCHILD, LORD, J. Exptl. Biol. *26,* 396 (1950).
17. LUNDBLAD, G., Acta Chem. Scand. *4,* 1075 (1950).
18. TISELIUS, A., Trans. Faraday Soc. *33,* 524 (1937).
19. SVENSSON, H., Arkiv Kemi, Mineral. Geol. *22 A,* no. 10 (1946).
20. DANIELSSON, C. E., and SANDEGREN, E., Acta Chem. Scand. *1,* 917 (1947).
21. HUNTER, A., and DOWNS, C. E., J. Biol. Chem. *157,* 427 (1945).
22. MONROY, A., Arkiv Zool. *40 A,* no. 21 (1948).
23. MACLEOD, J., J. Gen. Physiol. *34,* 705 (1951).
24. BÄCKSTRÖM, S., Unpublished results.

Tryckt den 13 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 20

Communicated 10 March 1954 by ARNE FREDGA and GUNNAR HÄGG

On the anionoid and cationoid substitutions of metallic derivatives of β-ketoesters and related compounds

By ARNE BRÄNDSTRÖM

Introduction

The mechanisms for the Claisen condensation of ethyl acetate to give ethyl acetoacetate and the alkylation of this compound have been the subject of many investigations and of an even greater number of speculations for well-nigh a century. The mechanisms proposed are so many and the investigations published so numerous that the subject is too immense even for a person specialized in it. In this paper the mechanisms given by different authors will not be discussed, most of them having been collected by HENECKA (15). Instead, the mechanisms for these two reactions will be considered in the light of some recent kinetic investigations by the present author and of the numerous experimental results of many other investigators, with HAUSER *et al.* being the foremost among them.

Possible mechanism

In both the Claisen condensation and the alkylation reaction the structure of the metal (usually sodium) compound is of great interest. In the mechanisms given by most investigators the sodium compounds of β-ketoesters are a priori assumed to be of the ionic type. As pointed out by BRÄNDSTRÖM (1) this is far from correct, but although the chelate character of many sodium compounds has been clearly demonstrated by SIDGWICK and BREWER (16) as early as 1925, very little has been done to use this to explain the Claisen condensation and the alkylation reaction.

JOHNSON (17) has assumed the existence of metal chelates in many reactions, but his theories have not been generally observed and accepted.

HAUSER (18) mentioned a chelate mechanism as an alternative to the ionic mechanism proposed by him for the Claisen condensation, but preferred the latter as being the more simple in the absence of proofs for a chelate mechanism. In later papers (19, 20), however, HAUSER prefers the chelate mechanism in the cases where magnesium and lithium compounds are used.

In the alkylation reaction, which is the more simple one to investigate, the reaction, in many cases, goes almost to completion with few side reactions. This and the possible formation of both O- and C-alkylation products makes it possible to choose between the ionic and the chelate mechanism from preparative results. This has been done by the present author (1) and the chelate mechanism so preferred for the C-alkylation reaction has been proven to be correct by means of kinetic measurements (2).

In the Claisen condensation the yields are usually not very high and the side reactions numerous. The yields obtained by the condensation thus offer no evidence of mechanisms (21). Since the Claisen condensation is very hard to investigate by kinetic methods the question if the reaction mechanism is of the ionic or the chelate type must, up to the present time, be considered as unsettled. In some recent papers the present author has, however, made a kinetic investigation of the acidolysis of ethyl diacetoacetate and found that in all reactions studied the ester or its anion was coordinated to the metal ion (3–7). Since the reaction medium used was rather polar the same results must be applicable to the reaction of alcohol with metal derivatives of β-ketoesters and related compounds in the less polar media used in the Claisen condensation of which it is the retrograde reaction. The mechanism of the Claisen condensation can thus be predicted by means of the kinetic results obtained from the acidolysis of ethyl diacetoacetate and the principle of microscopic reversibility (22) which enables the mechanism of the forward reaction to be known with as much accuracy as the mechanism of the reverse reaction.

The ionic mechanism

A reaction of a pure ionic type is defined as one in which the reacting substances are not coordinated to the cation (2). From the kinetic results it can be concluded that an ionic mechanism exists in some alkylation reactions (1, 2) but not in anionoid reactions (7).

In the ionic alkylation reaction the alkyl group can enter at different points of the mesomeric ester anion with different products as a result. The proportion of the different products can be predicted from the property of the mesomeric anion and the halide used. As the principles for the reaction of mesomeric anions are extensively discussed in most textbooks of theoretical organic chemistry such a discussion is omitted here.

It must, however, be pointed out that the proportion of the different products found may sometimes not be the same as that initially formed because of subsequent reactions. Such an example is given by EISENHAUER and LINK (23) and their results have been discussed by BRÄNDSTRÖM (8).

The chelate alkylation mechanism

As found by the present author (1, 2) a chelate alkylation mechanism of tautomeric substances easily explains the C-alkylation of β-diketones and β-ketoesters. This type of reaction has been carefully treated by the present author (1, 2). It must, however, be pointed out that in a nonpolar solvent the 2-covalent alkali compounds which may be present represent such an unusual coordination structure that any comparison with the properties of alkali compounds in dilute aqueous solutions is unsound. This may explain the rather curious tendency of the alkali metals to form 2-covalent compounds in the order $Li < Na < K$ (16).

In an alcoholic solution, on the other hand, the chelation tendency for the different alkali cations seems to follow the order expected $Li > Na > K$ (2).

The unusual coordination tendency of the alkali ions in a nonpolar solvent may result in an unexpectedly high difference in the alkylation velocity of compounds containing different alkali cations. — An increasing ionic radius results both in a higher concentration of the chelate and an increase of its reactivity. — Such an effect has been observed by BRÄNDSTRÖM (9) and by CRONYN whose results have been discussed by MARTELL and CALVIN (24) and by BRÄNDSTRÖM (10).

In this connection it may be pointed out that the suggestion by MARTELL and CALVIN (24) that the formation of the enol often is the rate-determining step in the alkylation must be incorrect. If it was correct the alkylation velocity would be the same if different alkyl halides were used, which is not in agreement with the experimental results well known to most organic chemists.

The anionoid reactions

The acidolysis of ethyl diacetoacetate has been investigated using various metal compounds as catalysts. From the kinetic results it can be concluded that there are two possible mechanisms for the reaction:

1. The reaction takes place through a complex consisting of the metal chelate of ethyl diacetoacetate to which the acetic acid is coordinated.
2. The reaction takes place between the ester coordinated to the metal cation and a free acetate anion.

The first type of reaction was found for metal cations with a high chelation tendency, in which case the metal compound probably is present largely in the chelate form.

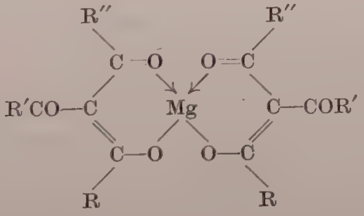
The second type, on the other hand, was found for the alkali compounds, which probably exist largely in an ionic form in the reaction mixture.

In no case studied was a pure ionic reaction found. This is of great interest since most anionoid reactions of preparative importance occur in solvents of a much lower polarity, with a corresponding higher tendency of the metal ion to form coordination compounds with the ketoester, than the acidolysis reaction. The mechanisms found for the acidolysis must hence be applicable to most anionoid attacks on the metal derivatives of tautomeric substances.

The acidolysis reaction

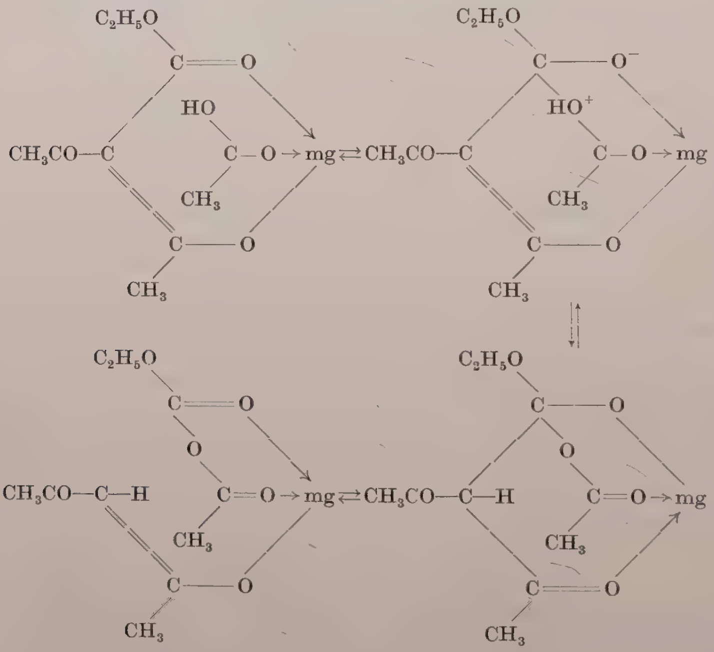
With magnesium oxide as a catalyst the acidolysis of ethyl diacetoacetate is of the first type and can be considered as an attack on the chelate by acetic acid coordinated to the cation (5, 7).

The chelate



has points of low electron density at the carbon atoms of the carbonyl groups. An anionoid attack of the chelate takes place at one of these points—which one depends on the substituents on the carbonyl groups. As found by the present author (5) the velocity of the magnesium oxide catalysed acidolysis is affected by substituents in the same way as the acid catalysed hydrolysis of esters. We can thus expect that the bulk of the R groups is the main factor determining the point of attack. In the reaction of a diethyl acylmalonate $R' = R'' = OC_2H_5$ and R = alkyl an attack at one of the carboxyl groups leads to the formation of an acylacetate (11), whereas an attack at the carbonyl group may give rise to an acyl ecxhange (12). The acyl exchange can be expected to be most important if R is a small group such as methyl, and in fact a rapid acyl exchange was observed when diethyl acetylmalonate was heated with *n*-butyric or *iso*butyric acid (12). A similar acyl exchange was observed between ethyl diacetoacetate and *n*-butyric acid (3).

The magnesium oxide catalysed acidolysis of ethyl diacetoacetate has been formulated in the following way (mg represents one equivalent of magnesium):



In this mechanism the mixed anhydride of acetic acid and monoethyl carbonate is assumed to be the primary reaction product which is rapidly decomposed into carbon dioxide and ethyl acetate.

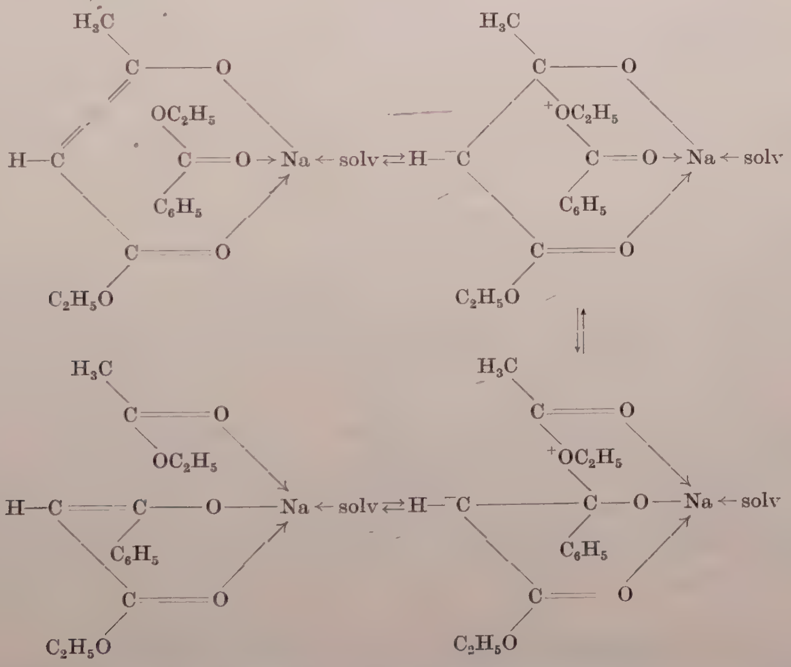
If the attack occurs at the carbonyl group the primary product is acetic anhydride, which if set free can function as a strong acetylation agent. In the reaction of a diethyl acylmalonate with the corresponding acid an attack at the carbonyl group results in an anhydride which may acylate the ketoester resulting from an attack at one of the carboxyl groups. The ethyl diacylacetate thus formed can then be acidolysed to give a diketone (13, 14). Since the relative extents of the carboxyl and carbonyl attacks of the diethyl acylmalonate determine the extent of the anhydride formed it can be concluded that a branching of the acyl group will result in a decrease in the formation of the diketone. The present author (13) has very approximately determined the extent of this diketone formation for the reaction of different diethyl acylmalonates with the corresponding acid RCOOH and found the yields: $R = CH_3CH_2$, 12 %; $R = CH_3CH_2CH_2$, 11 %; $R = (CH_3)_2CH$, 1 %; and $R = (CH_3)_3C$, 0 %. These are in good agreement with the yields that can be expected from the relative velocities for the acid hydrolysis of the esters of the corresponding acids.

It is of interest to note that the formation of the ethyl diacylacetate can also result from a direct acylation of the ethyl acylacetate by means of the diethyl acylmalonate (13, 3). Which of these mechanisms is the correct one is hard to establish (8).

Another very interesting acyl group exchange has been observed by McElvain and Weber (25) in the reaction of the sodium compounds of some β-diketones and β-ketoesters with an ester. With ethyl acetoacetate and ethyl benzoate the net reaction is

$$[CH_3COCHCOOC_2H_5]Na + C_6H_5COOC_2H_5 \rightarrow [C_6H_5COCHCOOC_2H_5]Na + CH_3COOC_2H_5 .$$

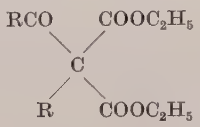
In this reaction we can expect that the sodium compound is present largely as the chelate which is soluble in the ester. From the fact that the reaction is much slower with diethyl malonate we can expect that the rate determining step of the reaction is an anionoid attack at the carbonyl group of the ketoester compound. The reaction can thus be formulated:

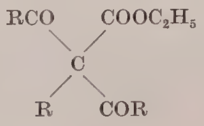


This mechanism explains why an attack preferentially takes place at the carbonyl group yielding a β-ketoester and not a β-diketone which would be the result of an attack at the carbethoxyl group.

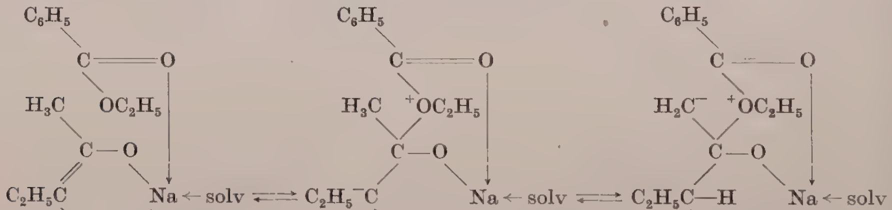
The mechanism proposed is very similar to the mechanism for the acyl exchange in the acidolysis of diethyl acetylmalonate.

As pointed out by the present author (3) the alkyl groups on the carbonyl carbon atoms of a chelate ring are points of high electron density. If the carbon atom between two carbonyl groups, which is usually the most important point of high electron density, is substituted by alkyl groups, the reactivity of this atom towards acylating agents is highly reduced. This can be seen from the fact that compounds of the types:





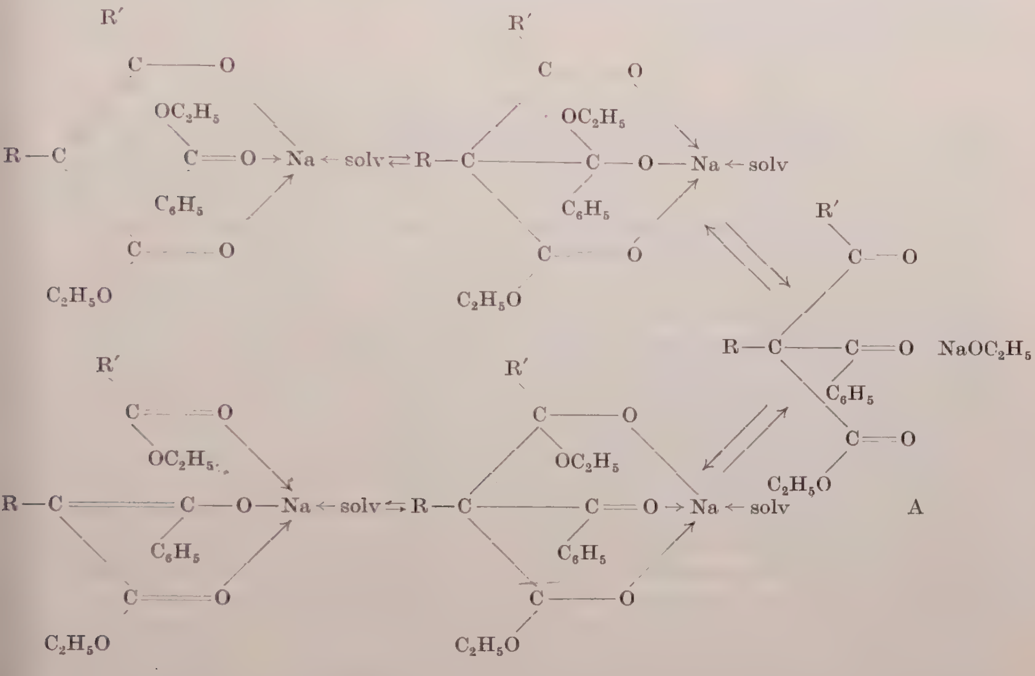
are cleaved by an alcoholic solution of sodium ethoxide even when cold (26). The anionoid reactivity of the methyl side-chain is, however, not hindered. The reaction of the sodium compound of ethyl ethylacetoacetate with ethyl benzoate may thus be formulated as:



The products isolated in this reaction were ethyl benzoylacetate and ethyl *n*-butyrate in agreement with the mechanism suggested.

When the methyl side-chain also was substituted by alkyl groups as in ethyl *iso*butyryl-*n*-butyrate the reaction products were ethyl ethylbenzoylacetate and ethyl *iso*butyrate. In this case the reaction obviously follows the same path as the reaction of ethyl acetoacetate and ethyl benzoate.

There are many arguments against the mechanism proposed by McElvain and Weber which was considered to involve at first a cationoid attack by the ester, thus:



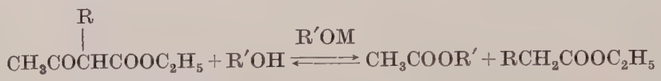
The intermediate A is very improbable if R is an alkyl group because alkylated diacylacetates are very unstable towards sodium ethoxide. If it exists it must have a very short life and the first cationoid attack would be the rate determining step, but in this case diethyl malonate would react much more readily than ethyl acetoacetate in this reaction since the former is much more rapidly alkylated and acylated than the latter (1).

If the acylation is the first step it should be possible to isolate ethyl diacylacetates from the reaction mixture, but no such compounds were found although many attempts were made to isolate them. The alcohol formed in some reactions is by no means a proof for the mechanism given by McElvain and Weber since this may have been formed by some of the numerous side reactions which are possible, e.g. the formation of dehydracetic acid.

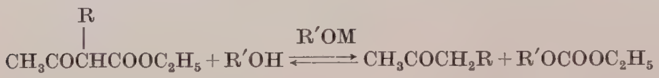
The mechanism for the Claisen condensation

The cleavage of β-ketoesters and related compounds with sodium ethoxide is of great interest as representing the reversal of the Claisen condensation. For the alcoholysis there are two mechanisms to be discussed. In one of them the ethoxide ion attacks the ester and in the other the alcohol attacks the anion or chelate of the ester. As pointed out previously, no pure ionic mechanism has been found for the acidolysis of ethyl diacetoacetate. Since the reaction medium in a Claisen condensation usually has a low polarity it seems very plausible to postulate that the same is valid for the alcoholysis reaction too.

The similarity between the acidolysis and the alcoholysis reactions is easily seen from the following equations:



$$CH_3CO\overset{\overset{R}{|}}{C}HCOOC_2H_5 + R'OH \overset{R'OM}{\rightleftharpoons} CH_3COOR' + RCH_2COOC_2H_5$$

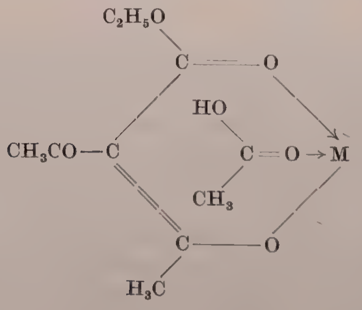


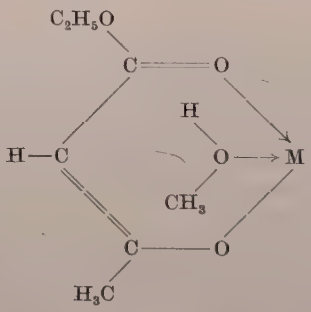
$$CH_3CO\overset{\overset{R}{|}}{C}HCOOC_2H_5 + R'OH \overset{R'OM}{\rightleftharpoons} CH_3COCH_2R + R'OCOOC_2H_5$$

With $R' = C_2H_5$ we have the two possible alcoholysis reactions and with $R' = CH_3CO$ we have the two acidolysis reactions, the acyl exchange and the decarbethoxylation reaction.

In applying the results obtained from the investigation of the acidolysis reaction to the alcoholysis reaction some interesting facts come to light.

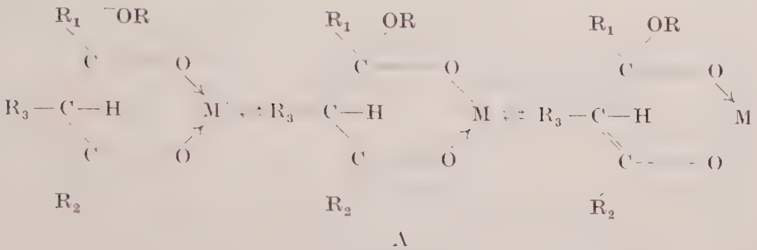
An attack on the chelate of ethyl diacetoacetate by the acetic acid has been observed only when the acetic acid can be coordinated to the metal ion in the chelate. Such a coordination tends to place the acetic acid in a position suitable for the reaction with the carbonyl groups of the chelate. If alcohol on the other hand is coordinated to a chelate it is not in a position suitable for the reaction with the carbonyl groups. This difference can be seen from the following formulae:





By a comparison with the acidolysis reaction we can thus expect the ester chelate to react neither with the alcohol coordinated to the chelate nor with alcohol not coordinated to it. From this it can be concluded that there are no mechanisms by which the alcoholysis can take place as an attack on the chelate by the alcohol.

The alcoholysis reaction must then take place by an attack on the ester by an ethoxide ion. Since the corresponding mechanism for the acidolysis reaction has been observed and found to involve the ester coordinated to the cation, the mechanism for the alcoholysis reaction can be formulated as



A

By means of the principle of microscopic reversibility (22) it can be concluded that the intermediate A, "transition state" (27), is the same in the alcoholysis as in the reversed reaction which is the Claisen condensation.

The mechanism for the Claisen condensation so deduced from the kinetic results of the acidolysis of ethyl diacetoacetate is the same as the chelate mechanism given by HAUSER (18–20). Since this mechanism is carefully treated by HAUSER in his numerous papers on condensation it will not be discussed in any detail. It is, however, interesting to note that the kinetic results have given a somewhat deeper insight in the mechanism than a mechanism deduced from preparative results only.

The fact that the chelate (and the anion) of a ketoester is resistent to an attack of alcohol is the most important new fact which is available from kinetic measurements only. This gives an interesting aspect on some well-known experimental results. Since the chelate is resistent to an attack of alcohol, it can be concluded that the formation of the chelate decreases the concentration of the ketoester available for the alcoholysis reaction, with a corresponding decrease in the velocity of this reaction. There is no simple correlation between the ease of condensation of an ester and the chelation tendency for the resulting ketoester, with the consequence that a decrease in the velocity of the alcoholysis reaction due to the chelation does not necessarily give rise to a corresponding decrease in the velocity of the condensation. Due to the reversibility of the reaction, a decrease in the alcoholysis reaction without the corresponding decrease in the condensation reaction will give rise to a better yield of the condensation product.

The effect of different cations and the polarity of the solvent—two important factors for the chelation—on the yields in the Claisen condensation has been very little investigated (20).

The effect of different alkyl groups R in the condensation of esters of the type $RCH_2COOC_2H_5$ has been extensively investigated but no acceptable theory (18) has been advanced to explain why ethyl *iso*valerate ($R = (CH_3)_2CH$) is not self-condensed by means of sodium ethoxide even under forcing conditions (28). The result is, however, easily explainable from the fact that an *iso*propyl group decreases the chelation tendency of β-ketoesters and related compounds to a very high extent. It may be mentioned that acetylacetonates do not give the usual coloured chelates with cupric and ferric ions when the 3-position is substituted with an *iso*propyl group but give typical chelates when substituted with a straight chain alkyl group.

In the condensation of ethyl *iso*valerate by means of sodium ethoxide the ethyl *iso*valeryl*iso*valerate that would result can accordingly not be transformed into the sodium chelate and is thus not withdrawn from the alcoholysis reaction. The yield of the condensation must then be very low, in good agreement with the experimental results.

The sodium (and potassium) compounds resulting from the self-condensation of esters are present in a chelate form, which can be seen from the fact that they are soluble in hydrocarbons such as benzene and toluene (29).

Summary

The mechanisms for the reaction of metallic derivatives of β-ketoesters and related compounds with cationoid and anionoid reagents have been discussed.

As an example of the attack by means of a cationoid reagent the alkylation has been mentioned. For this reaction both an ionic and a chelate mechanism has been observed.

The anionoid attacks by acids or their anions and by the ethoxide ion have been discussed.

The factors which make an attack by alcohol molecules improbable have been mentioned.

The mechanism for the Claisen condensation has been deduced and found in agreement with the chelate mechanism given by HAUSER.

An explanation for the fact that ethyl *iso*valerate is not self-condensed by means of sodium ethoxide has been given.

ACKNOWLEDGMENTS

The present paper is a summary of an investigation which has been carried out at the Institute of Chemistry, University of Uppsala, during the years 1950–1952 and finished at the Research Laboratory of Pharmacia Ltd., Uppsala.

To Professor ARNE FREDGA I wish to express my sincere gratitude for his kind interest in my work and for many stimulating discussions. I also wish to express my thanks to Professor GUNNAR HÄGG and to my colleagues for stimulating discussions.

I am also indebted to *Messrs. Pharmacia Ltd.* for permission to finish the work and to publish the results, and to Fil. Mag. SOLVEIG HANSEN for assistance in some experimental work.

The investigation has partly been supported by grants from the *Swedish Natural Research Council* and from *Bolidens Gruvaktiebolag* which are gratefully acknowledged.

REFERENCES

1. BRÄNDSTRÖM, A., Arkiv f. Kemi *6*, 155 (1953).
2. —— Ibid., *7*, 81 (1954).
3. —— Ibid., *6*, 201 (1953).
4. —— Ibid., *7*, 65 (1954).
5. —— Ibid., *7*, 89 (1954).
6. —— Ibid., *7*, 95 (1954).
7. —— Ibid., *7*, 103 1954.
8. —— Ibid., *7*, 29 (1954).
9. —— Ibid., *2*, 587 (1950).
10. —— Acta Chem. Scand. *7*, 223 (1953).
11. —— Ibid., *5*, 820 (1951).
12. —— Ibid., *5*, 972 (1951).
13. —— Ibid., *5*, 1413 (1951).
14. —— Arkiv f. Kemi *3*, 365 (1951).

15. HENECKA, H., Chemie der Beta-Dicarbonyl Verbindungen. Berlin 1950.
16. SIDGWICK, N. V., and BREWER, F. M., J. Chem. Soc. *1925*, 2379.
17. GILMAN, H., Organic Chemistry. John Wiley & Sons. New York 1944, p. 1879.
18. HAUSER, C. R., J. Am. Chem. Soc. *60*, 1958 (1938).
19. HAUSER, C. R., and WALKER, H. G., J. Am. Chem. Soc. *69*, 296 (1947).
20. HAUSER, C. R., and PUTERBAUGH, W. H., J. Am. Chem. Soc., *75*, 4756 (1953).
21. INGOLD, C. K., Structure and Mechanism in Organic Chemistry. London 1953, p. 792.
22. FROST, A. A., and PEARSON, R. G., Kinetics and Mechanism. New York 1953, p. 202.
23. EISENHAUER, H. R., and LINK, K. P., J. Am. Chem. Soc. *75*, 2044 (1953).
24. MARTELL, A. E., and CALVIN, M., Chemistry of the Metal Chelate Compounds. New York 1952, p. 393.
25. McELVAIN, S. M., and WEBER, K. H., J. Am. Chem. Soc. *63*, 2192 (1941).
26. DICKMANN, W., and WITTMANN, A., Ber. *55*, 3331 (1922).
27. Ref. 21 p. 794.
28. McELVAIN, S. M., J. Am. Chem. Soc. *51*, 3124 (1929).
29. BRÄNDSTRÖM, A., Acta Chem. Scand. *4*, 1608 (1950).

Tryckt den 9 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 21

Read 10 February 1954

Studies on synthetic growth substances

V. The configuration of the allyl-phenylacetic acid

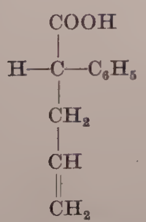
By Arne Fredga and Lars Westman

Among the numerous substances having plant growth regulating activity, the derivatives of *phenylacetic acid* have attracted comparatively small interest. Unlike the derivatives of 1-naphthylacetic acid, they show only moderate biological activity. An exception is the rather active α-allyl-phenylacetic acid, which has been investigated by Veldstra and van de Westeringh using the pea-test (1). This acid was also resolved into the optical antipodes, and it was found that the whole activity was due to the (+)-acid, the (−)-form being practically inactive.

Recently, Dr. B. Åberg, using the S-test (root growth of flax seedlings) has found a similar effect for the weakly active hydratropic (α-methyl-phenylacetic) acid. In this case the (−)-form is about 30 times more active than the (−)-form (2).

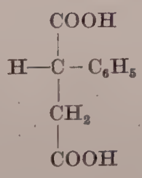
In the series of the *α-aryloxy-carboxylic acids*, steric effects have been studied by several authors and Matell has shown that those acids possessing higher auxin activity than their respective enantiomorphs belong to the D-series (3). For a better understanding of the mechanism of the growth-regulating activity it is essential to know if the same holds good for the substituted phenylacetic acids as well. In the case of the allyl-phenylacetic acid, the question could be settled in a rather simple way.

Ställberg-Stenhagen and Stenhagen found that (+)-methyl-allyl-acetic acid can be oxidised to methylsuccinic acid without loss of optical activity (4). In the same way the present authors could convert (+)-allyl-phenylacetic acid into (+)-phenylsuccinic acid. Recently, Dr. K. Pettersson has found that the latter acid is sterically connected to (+)-2-thienylsuccinic acid, which by treatment with Raney nickel could be converted into (+)-n-butylsuccinic acid (5). As this acid is known to have the D-configuration, (6), we obtain the following steric series:



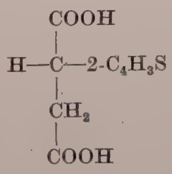
$$\begin{array}{c} COOH \\ | \\ H{-}C{-}C_6H_5 \\ | \\ CH_2 \\ | \\ CH \\ \| \\ CH_2 \end{array}$$

(+)



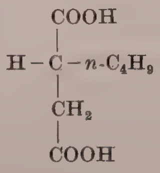
$$\begin{array}{c} COOH \\ | \\ H{-}C{-}C_6H_5 \\ | \\ CH_2 \\ | \\ COOH \end{array}$$

(+)



$$\begin{array}{c} COOH \\ | \\ H{-}C{-}2\text{-}C_4H_3S \\ | \\ CH_2 \\ | \\ COOH \end{array}$$

(+)



$$\begin{array}{c} COOH \\ | \\ H{-}C{-}n\text{-}C_4H_9 \\ | \\ CH_2 \\ | \\ COOH \end{array}$$

(+)

The formation of phenylsuccinic acid also affords a proof of the structure of the allyl-phenylacetic acid, showing that no migration of the double bond has taken place in the course of the synthesis.

The configuration of the hydratropic acid may still be open to question. Considering the structural similarity to allyl-phenylacetic acid, it is, however, very probable that the (+)-form, having the higher auxin activity, has the D-configuration. This question will be examined more closely.

Experimental

Allyl-phenylacetic acid. The racemic acid was prepared from ethyl phenylmalonate and allyl bromide according to PICKARD and YATES (7). It was purified by repeated fractionated crystallisations from the melt. The resolution was carried out with α-phenyl-ethylamine according to VELDSTRA *et al.* (1).

29.2 g acid and 20.1 g (−)-α-phenyl-ethylamine were dissolved in 200 ml of hot water. On cooling, 41 g salt were obtained. It was recrystallised from ethanol to constant activity, the rotatory power of the successive crops of the salt being measured in the same solvent. The data are in excellent accordance with those given by VELDSTRA *et al.* (1).

Crystallisation	2	3	4	5	6
ml ethanol	150	75	65	65	40
g salt obtained	22.3	16.3	13.7	10.5	9.0
$[\alpha]_D^{25}$ of the salt	+10.9°	+17.8°	+18.6°	+21.7°	+21.6°

The acid was liberated in the usual way and distilled *in vacuo* passing over at 160° (10 mm Hg).

0.2116 g acid: 11.11 ml 0.1075-*N* NaOH.
Equiv. wt. calc. 176.2, found 177.2.

0.2069 g dissolved in *benzene* to 9.98 ml: $2\alpha_D^{25} = +4.285°$. $[\alpha]_D^{25} = +103.3°$; $[M]_D^{25} = +182.1°$. The same solution was also measured at 20.00°: $2\alpha_D^{20} = +4.395°$. $[\alpha]_D^{20} = +106.0°$; $[M]_D^{20} = +186.8°$. — 0.2126 g dissolved in abs. *ethanol* to 10.00 ml: $2\alpha_D^{25} = +3.84°$. $[\alpha]_D^{25} = +90.3°$; $[M]_D^{25} = +159.1°$. — 0.2114 g dissolved in *acetone* to 10.00 ml: $2\alpha_D^{25} = +4.33°$. $[\alpha]_D^{25} = +102.4°$; $[M]_D^{25} = +180.5°$. — 0.2116 g dissolved in glacial *acetic acid* to 9.98 ml: $2\alpha_D^{25} = +3.88°$. $[\alpha]_D^{25} = +91.5°$; $[M]_D^{25} = +161.2°$.

The rotatory power in benzene is in good agreement with the value given by PICKARD and YATES ($[\alpha]_D = 103.19°$; no temperature reported) (7).

Oxidative degradation. 4.0 g of *racemic allyl-phenylacetic acid* were dissolved in 157 ml of glacial acetic acid. 31.4 g of finely powered potassium hydrogen sulphate and a solution of 7.1 g chromium trioxide in 10 ml water were added. On shaking, the temperature rose to 35°. The mixture was now heated to 65° (water bath) and kept at this temperature with occasional shaking for 15 minutes. After cooling and filtering, the acetic acid was distilled off *in vacuo* at a temperature not exceeding 70° (oil bath). The greenish-black residue was dissolved in sodium carbonate solution acidified with sulphuric acid and extracted with ether (200 ml in 5 portions). The solution was dried with anhydrous sodium sulphate, the ether evaporated and the oily residue kept in a desiccator over soda lime until the smell of acetic acid had disappeared. After maceration with benzene, there remained 0.9 g of a dry colourless powder. It was extracted with boiling benzene and recrystallised four times, alternately from water and ether + toluene. At last it was recrystallised twice from dilute hydrochloric acid (once with charcoal). Yield 0.15 g. It melted at 166–167° (capillary introduced at 163°, rise of temperature 2° per minute).

0.1097 g acid: 10.39 ml 0.1075-N NaOH.
Equiv. wt. calc. 97.1, found 98.2.

An authentic specimen of racemic phenylsuccinic acid melted under the same conditions at 167–168°, a mixed sample at 166–167.5°.

2.0 g of (+)-*allyl-phenylacetic* acid were oxidised in the same way as mentioned above, using 78.5 ml glacial acetic acid, 15.7 g of potassium hydrogen sulphate and 3.6 g chromium trioxide in 5 ml water. The temperature during the evaporation of the acetic acid was kept at 50–60°. After treatment with benzene, there remained 0.6 g of a colourless, dry powder. After two recrystallisations from ether + toluene and three from water (once with charcoal), there remained 0.20 g. M.p. 173–175° (decomposition).

0.1088 g dissolved in *acetone* to 10.00 ml: $2\alpha_D^{25} = +3.63°$. $[\alpha]_D^{25} = +166.8°$.

The rotatory power of the pure (+)-phenylsuccinic acid in *acetone* is $[\alpha]_D^{25} = +171.0°$ (8). The value obtained indicates a slight racemisation during the oxidation.

Summary

(+)-Allyl-phenylacetic acid, which has much stronger growth regulating activity than its enantiomorph, could be oxidised to (+)-phenylsuccinic acid, which is known to possess the D-configuration. It has previously been found that optically active α-(aryloxy)-carboxylic acids, which have higher biological activity than their respective enantiomorphs, belong to the D-series.

The authors are indebted to *AB Astra* for a generous supply of ethyl phenylmalonate. A grant from the *Swedish Natural Science Research Council* is gratefully acknowledged.

Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala, February 1954.

REFERENCES

1. Veldstra, H., and van de Westeringh, C., Rec. trav. chim. *70*, 1113 (1951).
2. Åberg, B., Private communication.
3. Matell, M., Stereochemical studies on plant growth substances, Diss., Uppsala 1953. Also in Kgl. Lantbruks-Högsk. Ann. *20*, 205 (1953).
4. Ställberg-Stenhagen, S., and Stenhagen, E., Arkiv Kemi, Mineral., Geol. 24 B, No. 9 (1947).
5. Pettersson, K. To be published.
6. Matell, M., Arkiv Kemi *5*, 17 (1952).
7. Pickard, R. H., and Yates, J., J. Chem. Soc. *95*, 1011 (1909).
8. Fredga, A., and Matell, M., Bull. soc. chim. Belg. *62*, 47 (1953).

Tryckt den 21 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 22

Communicated 28 October 1953 by G. De Hevesy and Karl Myrbäck

The uptake of Thorium B and Thorium C by yeast cells

By F. G. Sherman[1]

With 3 figures in the text

Introduction

When gaseous Thorium emanation is passed through blood, a large fraction of the daughter disintegration products have been shown to be associated with the red corpuscles (1, 2). In the present investigation, the uptake of Th B and Th C by yeast cells suspended in buffer at an acid pH and a neutral pH was studied. Evidence is presented which confirms and extends the observation of P. Genaud (3) on the conditions of uptake of heavy metal cations by yeast cells.

Methods and materials

Saccharomyces cerevisiae Hansen, was grown in a synthetic medium similar to that described by Atkin *et al.* (4), for 20–22 hours at 30°C. The cells were harvested by centrifugation and suspended, after washing, in 0.067 M phosphate buffer at a pH 6.8–6.9. In one series of experiments, oxygen which had passed over a radiothorium preparation having an activity of about one millicurie was led through four or five milliliters of yeast suspension for periods varying from one to five minutes (2). The "activated" cell suspension was shaken for twenty minutes in a thermostat at 30°C. The cells were separated from the activated buffer by rapid centrifugation and washed once with inactive buffer. The cells were resuspended in the same volume of inactive buffer and an aliquot of the cell suspension taken for Th C determination. The remainder of the cells were dried at 60° overnight and the dried cells formed into pellets, weighed and the activity of Th B determined. The activity of Th C in the buffer was determined from an aliquot pipetted onto an aluminum planchet. The remainder of the buffer was evaporated to dryness overnight at 60°C. The dried powder was formed into pellets having the same dimensions and approximately the same weight as those formed from cells for the determination of Th B.

In a second series of experiments, Th B and Th C activities were determined on cells which had been suspended in 0.067 M potassium acid phthallate buffer during exposure to Thorium emanation. Except where noted, the cells and buffer were treated as described above.

---

[1] Fellow in Radiological Research of the James Picker Foundation, recommended by the Division of Medical Sciences, National Research Council.

Table 1. Distribution of Th B between yeast cells and suspending buffer.

Experiment	Period of activation with Thoron min.	Incubation period min.	pH of the suspending medium	Counts/min/mg dry wt.		Counts/min/ mg dry wt. Cells/Buffer
				Cells	Buffer	
1	20	0	4.5	640	1095	0.58
	,,	60	,,	778	1310	0.59
		120	,,	960	1260	0.76
2	1	20	6.8	134	5.3	25.3
	,,	,,	2.3	0.15	1.26	0.12*
3	5	20	6.8	22.8	11.2	2.0
	,,	,,	2.3	19.5	183	0.11
4	3	60	6.5	53	15	3.3
	,,	,,	2.3	25	106	0.24

* Cells washed once with inactive buffer. Most of the activity was discarded.

Results

I. Uptake of Th B by yeast cells.

A. Direct determination of Th B.

The distribution of Th B between yeast cells and suspending buffer is shown in Table 1. Cells suspended in phosphate buffer at pH 4.5 have about 0.6 as much activity as the medium. The activities observed in the cells after incubation at 30°C for two hours suggest that Thorium B continues to be taken up by the cells at a reduced rate.

Cells activated while suspended in phosphate buffer at pH 6.8–6.9 take up much more Th B than they do when suspended in phosphate buffer at pH 4.5 or potassium acid phthallate buffer at pH 2.3. At pH 6.8, two to twenty-five times as much activity is associated with the cells as is present in the buffer. Cells activated in phthallate buffer at pH 2.3 take up only about 0.1–0.25 of the Th B present.

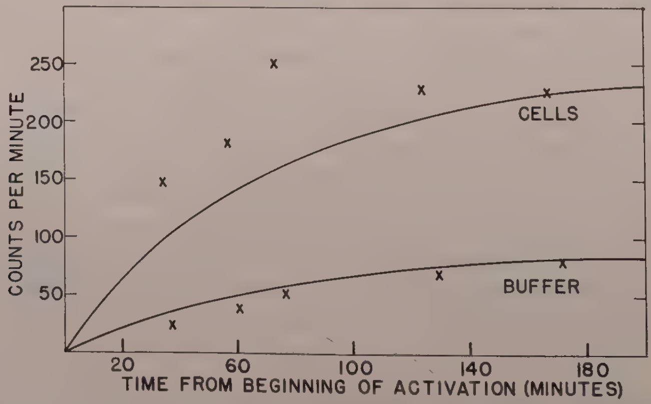


Figure 1. Thorium C activity of yeast cells activated at ph 6.8, and the separated buffer.

B. Distribution of Th B between cells and medium determined from Th C activity.

The passage of Thorium emanation through yeast suspended in phosphate buffer at pH 6.8–6.9 results in a large uptake of Th B by the cells as indicated by the increase in activity due to Th C with time (Fig. 1). The solid line shows the calculated increase in activity due to the formation of Th C from Th B (5). The points give the observed values for a typical experiment. The deviation of the observed activities from the calculated values are within the probable error of counting. In these experiments, the probable error of counting was large due to the necessarily small number of counts that could be taken in intervals of three to five minutes.

A comparison of the uptake of Thorium B by direct determination and by determination of the Thorium C activity at equilibrium is given in Table 2.

Table 2. Distribution of Th B between yeast cells and buffer as determined from the activity of Th C at equilibrium compared with the distribution of Th B between cells and buffer determined from Th B activity measurements. Yeast cells activated at pH 6.8–6.9.

Experiment	Method of determination	Period of activation min.	Incubation period min.	Activity observed		Ratio of activity Cells/Buffer
				Cells	Buffer	
2	counted Th B	1	20	134	5.3	25.3
3	,,	5	20	22.8	11.2	2.0
4	,,	3	60	53	15	3.3
9	counted Th C	1	20	67	15	4.4
12	,,	5	20	232	84	2.3

The determination of the distribution of Th B between cells and medium by counting the activity due to Th B gave values varying between 2.0 and 25.3. The ratio of Th B in cells and medium obtained by determining the Th C activity at equilibrium with Th B was 2.8 and 4.4 in two separate trials.

C. Effect of Pb and Bi on the uptake of Th B.

Yeast cells were incubated with dilute solution of $Pb(NO_3)_2$ – (3×10^{-4}M) to determine if it would be possible to saturate the yeast with Pb so that further uptake of Thorium B, which corresponds to Pb^{212} would be interferred with. It was found (Table 3) that cells treated with Pb or Bi before exposure to Thorium emanation took up much more Th B or products which decayed to Th B than did the untreated cells. (Thoron's first disintegration product is Th A, = 0.14 sec). In each instance that the yeast was incubated with Pb^{++} or Bi^{+++} before activation a larger total amount of activity was observed than in the nontreated cells originating from the same crop of yeast and activated on the same day. The ratio of activity in the cells pretreated with Pb^{++} and Bi^{+++} to the activity of the buffer ranged from 25.5 to 175.

II. Uptake of Thorium C.

The activity due to Th C in yeast cells and buffer of yeast activated in phthallate buffer at pH 2.3 is shown in Fig. 2. At pH 2.3, the activity associated with the cells falls off with time. The solid line shows the decay of Th C. The dashed line was

Table 3. Effect of incubating yeast cells with $Pb(NO_3)_2$ or $Bi(NO_3)_3$, before activation with Thoron, on the distribution of Th B between cells and buffer.

Experiment	Pretreatment of cells	Period of activation min.	Incubation period min.	Activity observed counts/min		Ratio of activity Cells/Buffer
				Cells	Buffer	
3*	None	5	20	22.8	11.2	2.0
3 a*	Incubated with $Pb(NO_3)_2$ 2 hours at 30° C (3×10^{-4} M)	5	20	272	1.5	175
10**	Same as 3 a	5	20	1160	45	26
11**	Incubated with $Bi(NO_3)_3$ 2 hours at 30° C (3×10^{-4} M)	5	20	255	10	25.5

* Counted Th B. ** Counted Th C.

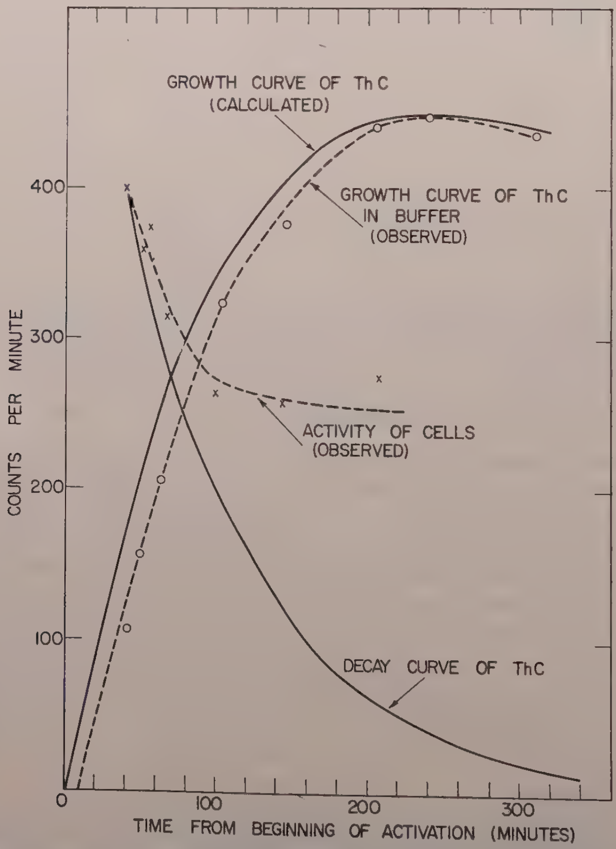


Figure 2. Thorium C activity of yeast cells activated at pH 2.3 and the separated buffer. Crosses indicate the observed activity in the cells. Open circles indicate the observed activity in the buffer.

drawn through observed values for the activity of the cells. The deviation of the observed activity of the cells from the decay curve of Th C indicated that some Th B was also taken up by the cells. Thus Th B furnished a diminishing but continuous supply of Th C during the period of observation.

The observed values for the activity in the medium increased steadily to a maximum about 200 minutes after activation. The solid line shows the calculated increase in activity due to the formation of Th C from Th B (5). Extrapolation of the observed values to the end of the activation period gives a value of zero for the Th C activity of the medium. This indicates that all of the Th C in the medium was taken up by the cells. The close correspondence of observed values for the activity in the medium with the calculated values corroborates this conclusion.

III. Removal of Th B and Th C from yeast.

A. Loss of Th B from yeast cells activated with Thorium emanation at pH 6.8.

An experiment was performed to determine if Th B could be removed from cells by washing and incubating cells with a) phosphate buffer at pH 6.8, or b) phthallate buffer at pH 2.1–2.3. Table four summarizes the results of two separate determinations. Washing the cells with buffer at pH 6.8, resuspending and incubating the cells in buffer of the same pH did not remove a measurable amount of Th B from the cells, (experiment 1 b and 2 b). Activated yeast cells were washed once with distilled water without subsequent incubation in experiment 1 a and 2 b.

In experiment 1 c and 2 c, after activation of the yeast cells in phosphate buffer at pH 6.8, the cells were washed with phosphate buffer, resuspended in phthallate buffer at pH 2.3 or 2.1 and incubated for 60 minutes at 30°C. Under these conditions Th B was actually lost from the cells to the buffer. Only one half to three quarters as much Th B remained on the cells as was found in the medium. Experiment 1d was similar to 1c and 2c except that $Pb(NO_3)_2 - (3 \times 10^{-4} M)$ was present in the phthallate buffer. The presence of Pb^{++} at this concentration in the buffer did not prevent the removal of Th B from the cells.

Table 4. The loss of Th B from yeast cells activated with Thoron for five minutes at pH 6.8. After activation the cells were washed with inactive buffer and resuspended in inactive buffer at the pH indicated. The resuspended cells were incubated with shaking at 30° C for 60 minutes.

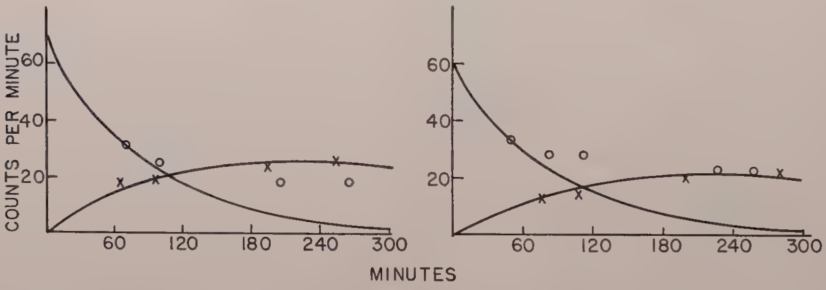
Experiment	pH of the suspending medium during incubation	Counts/min/mg dry wt.		Ratio of counts/ min/mg dry wt. Cells/Buffer
		Cells	Buffer	
1 a*	6.8	86	5.4	15.9
b	6.8	69	3.5	19.8
c	2.3	5.6	9.1	0.62
d**	2.3	5.4	7.1	0.76
2 a*	6.9	245	99	2.5
b	6.9	161	39	4.1
c	2.1	70	136	0.51
d	2.1	55	130	0.42

* Cells washed with distilled water.

** Cells incubated with phthallate buffer at pH 2.3 containing $Pb(NO_3)_2$ $(3 \times 10^{-4} M)$.

B. Loss of Th C from yeast cells activated in phthallate buffer at pH 2.3.

It was shown above (Fig. 2) that yeast cells suspended in phthallate buffer take up nearly all the Th C present in the medium in addition to a small amount of Th B. Figures 3a and 3b show the results of an experiment in which the yeast cells were activated while they were suspended in phthallate buffer at pH 2.3. After incubation in the activated buffer at 30°C for twenty minutes, the cells and active buffer were separated and the cells washed once with inactive buffer, resuspended and incubated with inactive buffer for ten minutes. The cells were again separated and the activity due to Th C was determined in the cells and buffer. Fig. 3a shows the Th C activity of cells which had been incubated with pthallate buffer at pH 2.3. The solid line shows the calculated decay of Th C. The open circles indicate the observed values. The deviation of the observed Th C activity of the cells from the theoretical decay curve for Th C indicates that some Th B remains associated with the cells which is furnishing a continuous but diminishing supply of Th C. The activity of the buffer increases to a maximum value at about 200 minutes after the beginning of the activation period. Extrapolation of the curve to the beginning of the incubation period with inactive buffer indicates that very little Th C was removed from the cells. The Th C activity of the buffer at equilibrium indicates that more than half of the Th B present on the cells was removed by the inactive buffer.



a.

b.

Figure 3. Thorium C activity of yeast cells activated in phthallate buffer at pH 2.3. a. Active cells incubated with inactive buffer (pH 2.3) for ten minutes. b. Active cells incubated with inactive buffer (pH 6.8) for ten minutes. Solid lines are the calculated decay and growth curves for Thorium C. Open circles indicate observed activity of the cells. Crosses indicate the observed activity of the buffer.

The results of an analogous experiment are shown in Fig. 3b. In this experiment the cells were activated in phthallate buffer at pH 2.3, and incubated in the inactive buffer for 20 minutes at 30°C. The cells were then separated by centrifugation, washed and incubated for ten minutes in inactive phosphate buffer at pH 6.8. Under these conditions about half of the Th B on the cells was removed by the buffer. Extrapolation of the buffer activity curve to the beginning of the incubation period indicates that there was no appreciable activity due to Th C. The deviation of the observed activity of the cells from the theoretical decay curve for Th C indicates that some Th B remains associated with the cells.

Discussion

The accumulation of Pb^{++} and other cations by plant tissues has been known since the observations of DEVAUX (6) in 1896 on the uptake of lead by *Elodea*. He also showed that the lead which accumulated in the cell walls of plants which had been placed in dilute solutions of $Pb(NO_3)_2$ could readily be displaced by replacing the lead solution with a solution containing potassium ions. This phenomenon was subsequently shown to be general (7). Cations of the heavy metals were found to be fixed by the cell walls of plants. These cations could be removed from the cell wall by washing the cells with a solution containing potassium, calcium or barium. Hevesy (8) showed that 95 % of the Th B in *Vicia faba* was removed when the plants were placed in $Pb(NO_3)_2$ (10^{-2}N) for 24 hours. Immersion of plants containing Th B in 10^{-3}N $Cd(NO_3)_2$ resulted in the loss of 62 % of the Th B; under the same conditions, immersion in 10^{-2}N $NaNO_3$ resulted in the removal of only 24 % of the Th B. Of the ions investigated, only cupric nitrate was able to displace Th B in the plants as effectively as lead did. These data led to the conclusion that lead exists in the plant in the form of a dissociable salt which is only slightly soluble. GENEVOIS (9) suggested an explanation of Devaux's observation on the basis that at least one of the anion species in the system is completely insoluble while the others are soluble. Monovalent cations will be displaced by soluble divalent or trivalent cations even at high dilutions. Monovalent cations will displace those of higher valence when the former are present in excess.

These observations were extended to apply to yeast by P. GENAUD (3, 10). He showed that yeast took up lead rapidly from dilute solutions and that a stoichiometric quantity of potassium and calcium was displaced by the lead ion. Genaud showed that the Pb^{++} was toxic only when it was allowed to remain in the cells for several days. During periods of a few hours there was no measurable effect on the intensity of fermentation and respiration. These experiments demonstrated that ion exchange is exhibited by normal cells and that it is completely reversible.

The observations reported in the present paper may be visualized in terms of an ion exchange process, similar to that described by GENAUD (3) taking place in the outer compartment (11) of the yeast cell. Both Th B and Th C are taken up by the cell at neutral or slightly acid pH (Fig. 1). At acid pH (2.3) in phthallate buffer, Th B is replaced by hydrogen ions, but Th C is not (Fig. 2 and Table 4). If an ion exchange mechanism is operating, the results suggest that Th B, which corresponds to Pb^{212}, is present as an ionic species which has a smaller net residual charge than does Th C (Bi^{212}).

The writer wishes to express his appreciation to Professor G. HEVESY for his help and encouragement in the conduct of this investigation.

SUMMARY

1. The distribution of the solid disintegration products of Thorium emanation between yeast cells and the suspending medium was determined at pH 6.8 and pH 2.3.

2. At pH 6.8 both Th B and Th C was taken up by the cells.

3. Th C was taken up by the cells at pH 2.3. At this pH only a small amount of Th B became associated with the cells.

4. Washing cells activated at pH 6.8 with buffer at pH 2.3 removed a large fraction of the Th B activity. It was not possible to remove Th C from the cells by washing with buffer in the acid or neutral range.

5. Incubation of the cells with $Pb(NO_3)_2$ or $Bi(NO_3)_3$ (3×10^{-4}M) before exposure to Th emanation did not prevent the uptake of Th B.

Department for Research in Organic Chemistry, University of Stockholm, Sweden.
Present address: Department of Biology, Brown University, Providence 12, R.I., U.S.A.

LITERATURE

1. Hevesy, G., 1951, Arkiv f. Kemi, *3*, 425.
2. Alexander, E., 1952, Arkiv f. Kemi, *4*, 363.
3. Genaud, P., 1929, C. R. Acad. Sc., *189*, 591.
4. Atkin, L., P. Gray, W. Moses, and M. Feinstein, 1949, Biochim. et Biophys. Acta, 3, 692.
5. Hevesy, G. and F. A. Paneth, 1938, A Manual of Radioactivity, Oxford University Press, London.
6. Devaux, H., 1896, Mem. Soc. Sc. Phys. et Nat. de Bordeaux, 5^me^ série.
7. ——, 1901, C. R. Ac. Sc., *132*, 717.
8. Hevesy, G., 1923, Biochem. J., *17*, 439.
9. Genevois, L., 1925, P. V. Soc. Sc. Phys. Bordeaux, 24, XII.
10. ——, 1950, Rev. Fermentations et Ind. Aliment., *6*, 88.
11. Conway, E. J. and M. Downey, 1950, Biochem. J., *47*, 347.

Tryckt den 21 april 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 23

Read 13 January 1954

The linkages between FMN and the protein of the "old yellow enzyme" studied by means of fluorescence measurements

By Hugo Theorell and Agnar P. Nygaard[1]

With 1 figure in the text

When flavin mononucleotide (FMN) and protein combine to form the old yellow enzyme (O.Y.E.), the absorption maximum of FMN shifts from 445 mμ to 465 mμ (1), and the fluorescence of the free FMN disappears. It would be possible to employ this absorption shift for the study of the equilibria and kinetics of the protein-FMN system in the same way as Theorell *et al.* (2, 3) have already used an absorption shift for the study of the DPNH-alcohol dehydrogenase (ADH) system. However, the spectrophotometric study of the DPNH-ADH system could not be carried out at concentrations of DPNH lower than 10^{-6} M, and a study of the old yellow enzyme on the basis of changes in light absorption could be calculated to have the same practical limitation.

The disappearance of the fluorescence of FMN was used by Theorell (4) for the titration of the protein of the O.Y.E. Since more sensitive and accurate instruments for the measurements of fluorescence can now be constructed, it seemed promising to study the reaction velocities and equilibria in flavin-protein systems with the aid of fluorescence measurements. We used a photomultiplier-amplifier-recorder system that will be described in another communication. It was found that the sensitivity of fluorescence measurements could easily be brought to exceed that of spectrophotometry by at least 1000 times, so that concentrations of FMN as low as 10^{-9} M, or even lower, could be used for quantitative determinations.

Experiments by Theorell in 1934 (1) with a crystallized preparation of the old yellow enzyme indicated strongly that one molecule of FMN combines with one molecule of protein:

$$\text{FMN} + \text{Protein} \underset{k_2\,(\text{sec}^{-1})}{\overset{k_1\,(\text{liters} \times \text{mole}^{-1} \times \text{sec}^{-1})}{\rightleftarrows}} \text{FMN} \cdot \text{Protein}\ (=\text{Enzyme})$$

Now, it is believed that FMN and protein are combined through two chemical bonds, the NH-(3) imino group and the phosphate group of FMN being involved (5). If the two linkages interact in such a way that the disruption or formation of one linkage greatly favours the disruption or formation of the other, one may expect the following relations to be valid:

---

[1] Fellow, Norwegian Research Council.

The rate of dissociation ("off reaction") of the enzyme should be proportional to the enzyme concentration

$$-dc/dt = k_2 \times [\text{FMN-Protein}].$$

The rate of combination between FMN and apoenzyme (the "on reaction") should follow the equation:

$$-dc/dt = k_1 \times [\text{FMN}] \times [\text{Protein}].$$

In a great number of experiments at different pH and salt concentrations, we have investigated both the on and the off reactions. In calculating the reaction rates we have used both the initial velocities at $t = 0$ and the integrated forms of the complete equation:

$$-dc/dt = k_2 \times [\text{FMN-protein}] - k_1 \times [\text{FMN}] \times [\text{protein}] \text{ (off reaction), or}$$

$$-dc/dt = k_1 \times [\text{FMN}] \times [\text{protein}] - k_2 \times [\text{FMN-protein}] \text{ (on reaction).}$$

It was found that k_2 and k_1 are true constants, and the equilibrium constant, calculated from the equation $K = k_2/k_1$, was found to agree with the values of K obtained in direct determinations of the dissociation equilibria at varied dilutions. The assumption of a strong interaction between the two FMN-protein bonds thus seems to be valid.

Quenching effects of salts on fluorescence

The fluorescence of riboflavin disappears both in acidic and alkaline solutions, in the latter case following a dissociation curve with $pK' = 10.2$ (6). More recently, WEBER (7) has discovered the quenching of the fluorescence of riboflavin by a number of organic compounds, silver ions and iodide ions. We have now found that halogen ions quench the fluorescence of FMN in the order $I^- > Br^- > Cl^-$. F^- gave about the same quenching effect at Cl^-. Whereas Br^- and Cl^- appeared to form dissociating compounds in the ratio 1:1 with FMN, the reaction with I^- seemed to be more complicated. This is in accordance with WEBER's (7) results using riboflavin and iodide. Sulphate, phosphate and acetate ions did not quench. $CaCl_2$, NaCl, KCl and NH_4Cl all give the same quenching effect. The results are recorded in Fig. 1. It was obviously important to correct the deflections of the recorder for the quenching effect of chloride ions. This was done by multiplying by the quenching factor $(Q.f.) =$ Fluorescence in water/Fluorescence in salt.

Effects of pH and chloride concentrations on O.Y.E.

All the following results were obtained at 23.5° C.

In pure water the recombination of FMN and O.Y.E.-protein gave a velocity constant k_1 of 10^6 liters $\times$ mole^{-1} $\times$ sec^{-1}. This figure is approximate, since the protein is unstable in high dilutions in water. When O.Y.E. was diluted to 10^{-6}—2×10^{-9} M, no increase of fluorescence was observed. This means that the dissociation rate constant, k_2, is unmeasurably small in water. From measurements of k_2 in salt solutions we may conclude that the equlibrium constant in water is below 10^{-12} M.

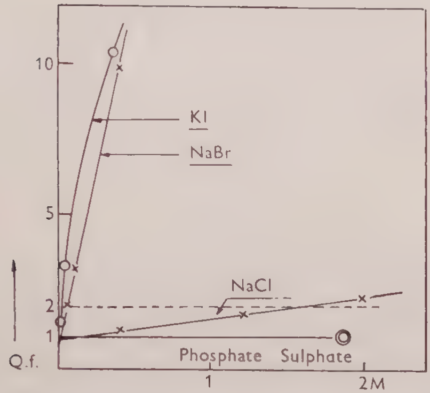


Fig. 1. The quenching effect of different salts on the fluorescence of FMN. $T = 23.5°$ C. $Q.f.$ = Fluorescence in water/Fluorescence in salt. - - - - Level for 50 % quenching ($Q.f. = 2$).

Changes in pH and the addition of chloride ions have a profound influence on the off reaction and equilibrium constant. On the acid side of pH 6, the following relationship was found to obtain:

$$k_2 = 1000 \times [H^+] \times [Cl^-] \text{ at } 23.5° \text{ C.}$$

The combination rate (k_1) is far less influenced by chloride ions. Concentrations of < 0.1 M had no appreciable effect; at higher concentrations a moderate decrease of k_1 was observed. An idea of the influence of hydrogen ions on k_1 in the acid range may be obtained from the following results in dilute acetate buffers:

$$\text{pH } 5.7\text{: } k_1 = 0.3 \times 10^6\text{; pH } 4.9\text{: } k_1 = 0.035 \times 10^6\text{;}$$

$$\text{pH } 4.07\text{: } k_1 = 0.003 \times 10^6 \text{ liters} \times \text{mole}^{-1} \times \text{sec}^{-1}.$$

In the alkaline range, between pH 8.03 and 10.36, we have observed an interesting relationship between k_2 and the quenching factor. Using a 0.1 M glycine buffer and adding NaCl to 0.4 M to give convenient dissociation velocities, we found k_2 to be proportional to the quenching factor:

$$k_2 = 17.5 \times 10^{-4} \times Q.f.$$

k_1, k_2 and K remained essentially constant from pH 8 to 9 in these solutions ($k_1 = 0.2 \times 10^6$; $k_2 = 25 \times 10^{-4}$; $K = 1.25 \times 10^{-8}$). Above pH 9 k_2 and K increased, k_1 decreased.

The effects of phosphate and other anions

The on reaction is strongly inhibited by phosphate, the inhibition increasing with the phosphate concentration. Thus, in phosphate buffers of pH 5.7 and ionic strengths 0.02 and 0.1, the reaction rates were found to decrease twofold and fivefold, respectively. Strangely enough, chloride counteracted the inhibiting effect of phosphate.

Phosphate and also sulphate increased the off reaction, but the effect of these anions was about 50 times weaker than the effect of chloride. Acetate appeared to have no dissociating effect on the FMN-protein complex. The dissociation rate in 0.4 M sodium chloride of pH 5.7 was at least five hundred times faster than in sodium acetate of the same ionic strength and pH.

Discussion

The inhibition of the on reaction by phosphate may be due to a competition with the phosphate ester of FMN for an active site of the protein. It is entirely possible that this may be a general effect on coenzymes containing phosphate esters.

Chloride may increase the off reaction by forming a salt with the $\overset{|}{\underset{/}{\mathrm{N}}}\mathrm{H}$ (3) group of FMN. This is supported by the fact that the hydrolysis of the enzyme is proportional to the quenching of FMN in the alkaline range. The combination of this imino group with the protein is believed to cause the quenching (5).

The facts that chloride mainly increases the off reaction and has a relatively small effect on the on reaction, whereas phosphate, on the opposite, mainly inhibits the on reaction and has a small effect on the off reaction, could have the following explanation. The dissociation of the enzyme is initiated by the cleavage of the bond between the imino group $\overset{|}{\underset{/}{\mathrm{N}}}\mathrm{H}$ (3) of FMN and the protein. On the other hand, the combination of FMN and protein is initiated by the phosphate group of FMN being attached to the protein.

Preliminary experiments together with R. BONNICHSEN, using the fluorescence of DPNH for the measurements, indicate that chloride ions have strong effects on the reaction velocities in the DPN + ADH + ethanol, as well as in the DPNH + ADH + acetaldehyde system. Small additions of chloride (0.01–0.1 M) increase the velocity in the first system; greater concentrations inhibit.

THEORELL, BONNICHSEN and CHANCE (2, 3) have shown that the dissociation rate of DPNH-ADH may be the rate-limiting reaction in the oxidation of alcohol. It is therefore of importance that this reaction is stimulated by chloride ions. This effect may be of general importance in splitting coenzyme-protein compounds.

In the second system, DPNH + ADH + acetaldehyde, chloride causes only inhibition, increasing with concentration. These experiments indicate that chloride may have different effects on the reduced and the oxidized form of the enzyme. This is important, since it means that *chloride ions may have an influence on the redox potential of the enzyme*. It appears likely that this may be a general phenomenon, valid for flavin[1] and pyridine-nucleotide proteins, and perhaps also for other coenzyme-protein systems. The dissociating effect of chloride has been used in a related class of substances, the nucleoproteins. As long as thirty years ago HAMMARSTEN (8) produced protein-free nucleic acids by the addition of sodium chloride to nucleoproteins.

It should further be remembered that chlorides have recently been found to promote the dissociation of some haemoproteins, peroxidase (9), haemoglobin, myoglobin, catalase (10), and that chloride ions in cooperation with hydrogen ions enter into the cytochrome c molecule, presumably between the iron atom and the nitrogen atoms of the protein part (11).

---

[1] A study of FAD-containing enzymes is being undertaken by Dr. O. WALAAS and Dr. E. WALAAS at this Institute.

From these examples it is evident that chloride ions have many interesting effects on conjugated protein systems. The functional importance of these ions may turn out to be comparable to that of the hydrogen ions, and it is understandable that they are kept at a constant level in the living cells.

ACKNOWLEDGMENT

The expenses connected with this investigation have been covered by grants from the Rockefeller Foundation and Knut och Alice Wallenbergs Stiftelse. Brewers yeast for the preparation of O.Y.E. was kindly supplied by Hamburger Bryggeriet, Stockholm.

One of us (A.P.N.) wishes to express his gratitude to A/S Freia Chokolade Fabriks arbeidsfond for ernaeringsforskning for a personal grant.

The Medical Nobel Institute, Biochemical Department.

REFERENCES

1. Warburg, O. and Christian, W., Biochem. Z. *254*, 438 (1932). Theorell, H., *ibid. 278*, 263 (1935).
2. Theorell, H. and Bonnichsen, R., Acta Chem. Scand. *5*, 1105 (1951).
3. Theorell, H. and Chance, B., Acta Chem. Scand. *5*, 1127 (1951).
4. Theorell, H., Biochem. Z. *290*, 293 (1937).
5. Kuhn, R. and Boulanger, P., Ber. *69*, 1557 (1936).
6. Kuhn, R. and Moruzzi, G., Ber. *67*, 888 (1934).
7. Weber, G., Biochem. J. *47*, 114 (1950).
8. Hammarsten, E., Biochem. Z. *144*, 1 (1924).
9. Maehly, A. C., Biochim. et Biophys. Acta *8*, 1 (1952).
10. Lewis, U. J., J. Biol. Chem. In press.
11. Boeri, E., Ehrenberg, A., Paul, K. G., and Theorell, H., Biochim. et Biophys. Acta *12*, 273 (1953).

Tryckt den 13 maj 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 24

Communiqué le 27 Mai 1953 par G. de Hevesy et Karl Myrbäck

Incorporation du ^{14}C dans les graisses et le glycogène du foie chez des souris irradiées ayant reçu une injection de glucose marqué par du ^{14}C

Per M. Lourau-Pitres

Avec 4 figures dans le texte

On sait que l'irradiation générale du corps préserve le glycogène du foie pendant le jeune (1–2–3–4) et l'on suppose que deux mécanismes concourent à produire cet effet: l'augmentation du catabolisme protidique (5) et la diminution d'utilisation des glucides (6).

Plusieurs auteurs ont tenté de préciser le point d'attaque des rayons sur le catabolisme des glucides. Les observations de Dubois, Cochran et Doull (7) suggèrent que l'irradiation inhibe l'une des réactions oxydatives du cycle de Krebs; pour Stocken (3), l'inhibition serait plus précoce. D'autre part, les essais faits pour identifier l'enzyme inhibé par les rayons n'ont donné que des résultats négatifs (8). On ignore donc encore comment les rayons préservent le glycogène du foie pendant le jeune.

Un autre problème lié au précédent et que nous avons traité dans ce travail est de savoir comment des animaux irradiés utilisent le glucose non point en période de jeune, mais lorsqu'ils sont normalement alimentés. On sait que le glucose qui pénètre dans la circulation par voie digestive est rapidement métabolisé. Une partie est oxydée directement par les tissus périphériques, 40 p. 100 selon Chaikoff (9) et Drury (10); le reste est retenu par le foie; une petite fraction, 3 p. 100, est incorporé dans le glycogène, une fraction plus importante, 35 à 50 p. 100, dans les graisses (11).

Trois constituants sont donc, à notre point de vue, d'un intérêt primordial: le CO_2 exalé d'une part, et de l'autre, le glycogène et les graisses du foie.

Hevesy et Forssberg (6) ont étudié l'exalation du CO_2, et ils ont constaté qu'elle était diminuée après l'irradiation; dans ce travail ce sont les graisses et le glycogène du foie qui ont retenu notre attention. Nous avons cherché comment des animaux normalement alimentés, mais dont le glucose sanguin a été marqué par une petite quantité de glucose contenant du ^{14}C uniformément réparti dans toute la molécule incorporent le ^{14}C dans les graisses et le glycogène du foie.

I. Conditions expérimentales

Nous avons travaillé sur des souris mâles adultes (25 à 30 g) réparties en lots de 4 dont les organes ont été mélangés et analysés ensemble. Les points qui figurent sur les courbes représentent les valeurs trouvées sur le mélange de 4 organes. Les animaux sont alimentés sauf la courte période qui sépare l'irradiation de la mort.

Les différents lots ont été répartis entre deux groupes d'animaux, un groupe de témoins et un groupe qui reçoit une irradiation générale par les rayons X dans les conditions décrites en 6. Immédiatement après l'irradiation témoins et irradiés reçoivent une injection sous cutanée de 0,1 ml d'une solution isotonique de NaCl contenant environ 350 γ de glucose marqué ayant une activité voisine de 3 à 10 micro curies.

Les animaux sont tués par décapitation, le sang recueilli sur héparine et fluorure, le foie prélevé et congelé.

Le *sang* a été desséché sous vide et réduit en poudre. Une partie a été auparavant mélangée à 0,1 ml de HCl 2N de manière à éliminer le CO_2.

Les *graisses* du foie ont été extraites à partir de la pulpe fraîche broyée au mortier au moyen de deux extractions de deux heures chacune, la première par un mélange

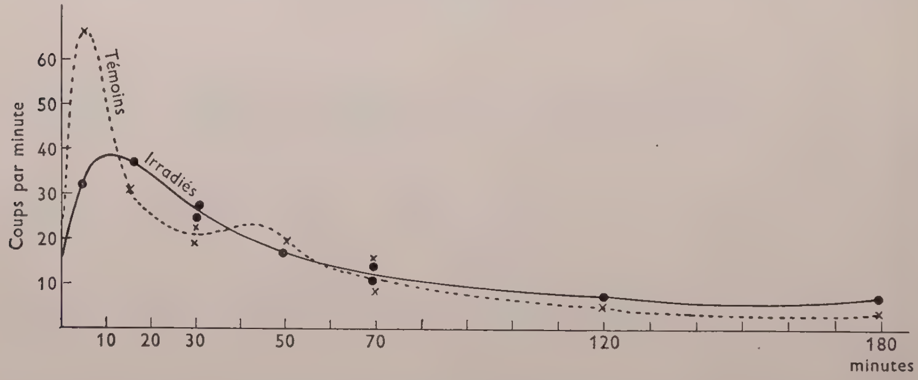


Fig. 1 a. Radioactivité du sang de souris témoins et de souris irradiées avec 2000 r après l'injection de glucose marqué par du ^{14}C.

Rapport des surfaces entre 0 et 70 mn: $\frac{\text{Irradiés}}{\text{Témoins}} = 1{,}01$.

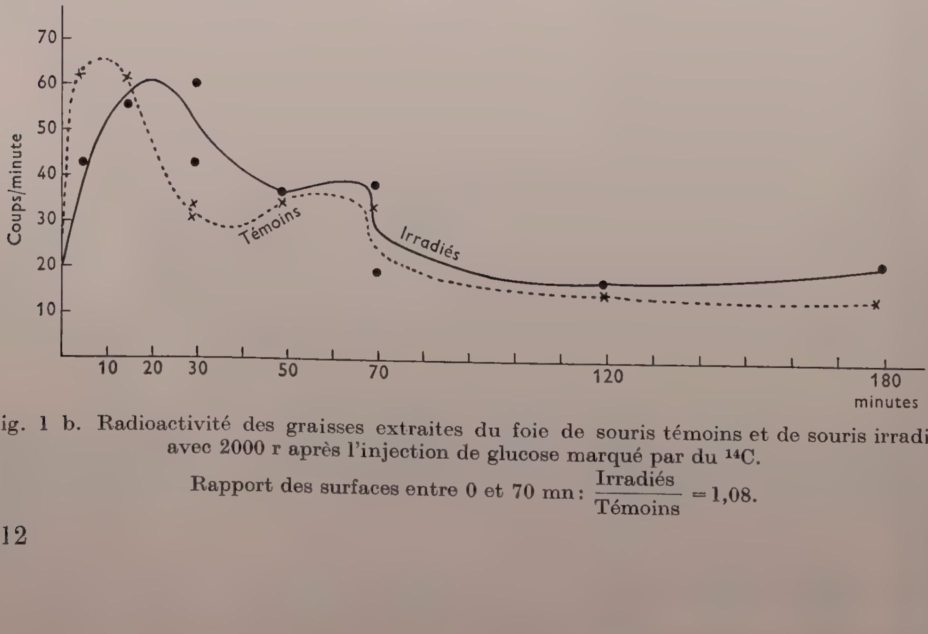


Fig. 1 b. Radioactivité des graisses extraites du foie de souris témoins et de souris irradiée avec 2000 r après l'injection de glucose marqué par du ^{14}C.

Rapport des surfaces entre 0 et 70 mn: $\frac{\text{Irradiés}}{\text{Témoins}} = 1{,}08$.

éther-éthanol (1/3) la deuxième par un mélange méthanol-chloroforme (1/1). Les filtrats sont mélangés, distillés sous vide, les graisses reprises par de l'éther de pétrole, séchées et pesées.

Le *glycogène* du foie a été extrait par KOH 33 p. 100 à partir du résidu d'extraction des graisses. On hydrolyse à froid pendant 24 h, on centrifuge pour éliminer un précipité brun non radioactif donnant la réaction du fer, on précipite le glycogène par deux volumes d'éthanol à 96°, puis on le redissous dans une petite quantité d'eau. On titre le glycogène par la méthode de Somogyi sur un partie aliquote de cette solution aqueuse, et on précipite le reste du glycogène par deux volumes d'éthanol. On lave le précipité à l'alcool, à l'éther, et on le sèche.

La *radioactivité* a été mesurée sur des échantillons de 100 mg pour le sang et la graisse, de 40 mg pour le glycogène. L'activité est exprimée en nombre d'impulsions par minutes (figures 1 et 2) ou en p. 100 de la quantité de ^{14}C injecté retrouvée dans la totalité des graisses et du glycogène (figure 3).

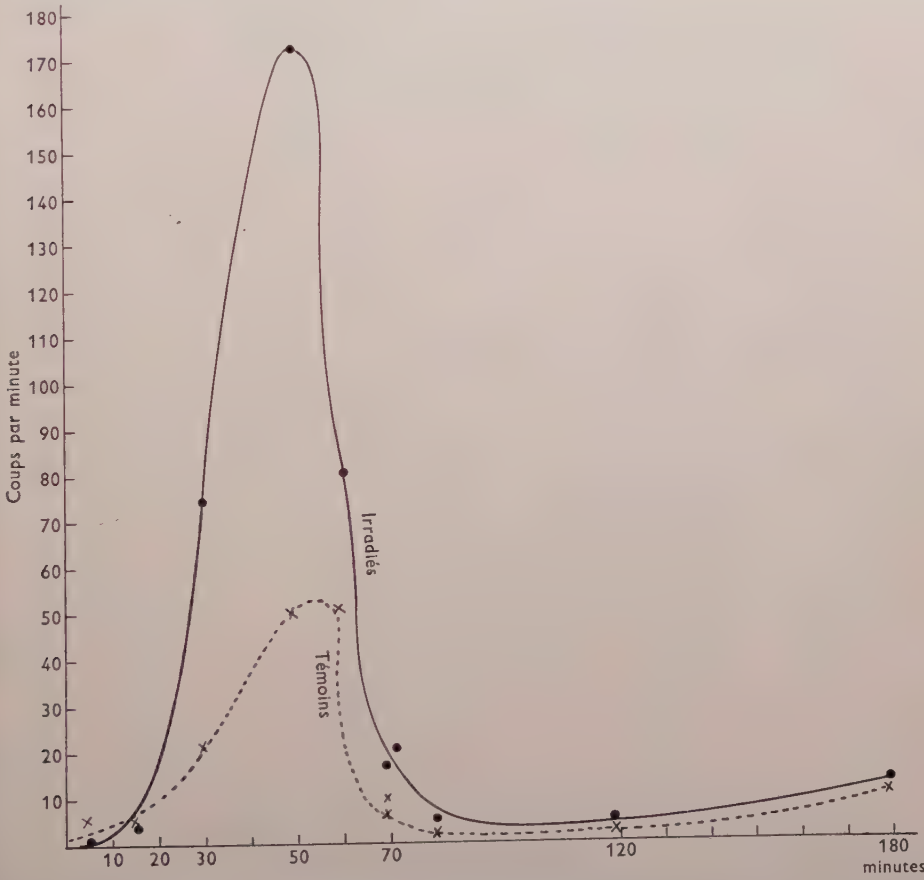


Fig. 1 c. Radioactivité du glycogène du foie de souris témoins et de souris irradiées avec 2000 r après l'injection de glucose marqué par du ^{14}C.

Rapport des surfaces entre 0 et 70 mn: $\frac{\text{Irradiés}}{\text{Témoins}} = 3{,}35$.

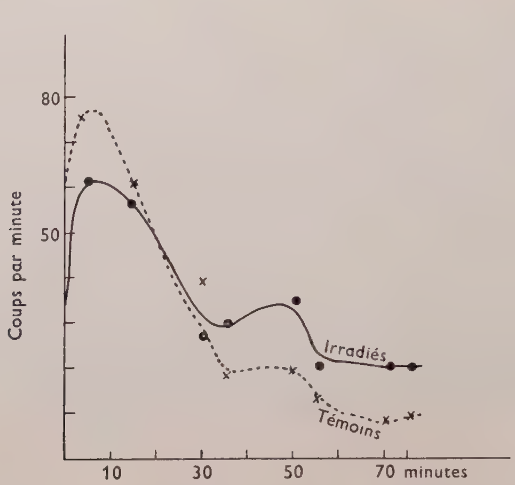


Fig. 2 a. Radioactivité du sang de souris témoins et de souris irradiées avec 2500 r après l'injection de glucose marqué par du ^{14}C.

Rapport des aires entre 0 et 70 mn : $\frac{\text{Irradiés}}{\text{Témoins}} = 0,99$.

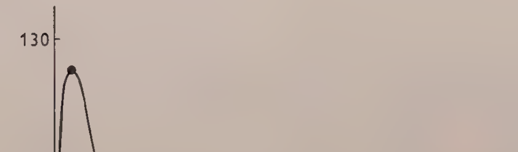


Fig. 2 b. Radioactivité des graisses extraites du foie de souris témoins et de souris irradiées avec 2500 r après l'injection de glucose marqué par du ^{14}C.

Rapport des aires entre 0 et 70 mn : $\frac{\text{Irradiés}}{\text{Témoins}} = 1,57$.

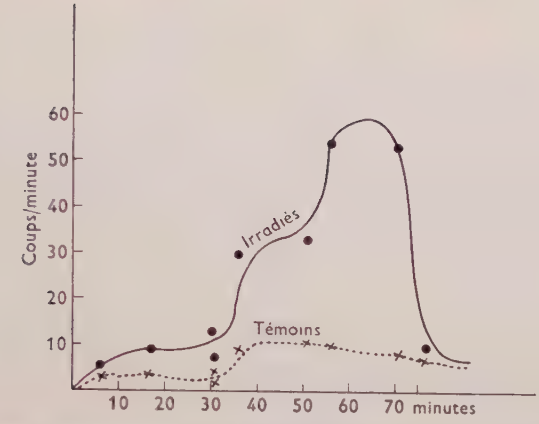


Fig. 2 c. Radioactivité du glycogène du foie de souris témoins et de souris irradiées avec 2500 r après l'injection de glucose marqué par du ^{14}C.

Rapport des aires entre 0 et 70 mn: $\frac{\text{Irradiés}}{\text{Témoins}} = 3{,}4$.

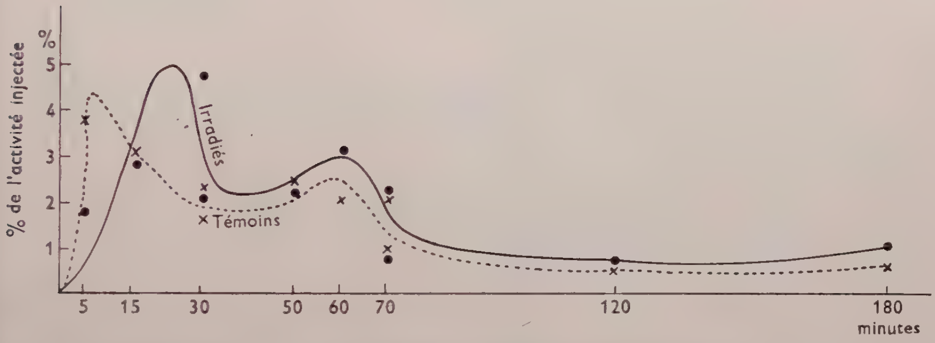


Fig. 3 a. Radioactivité (en p. 100 de l'activité injectée) des graisses extraites du foie de souris témoins et de souris irradiées avec 2000 r après l'injection de glucose marquè par du ^{14}C.

Rapport des aires entre 0 et 70 mn: $\frac{\text{Irradiés}}{\text{Témoins}} = 1{,}21$.

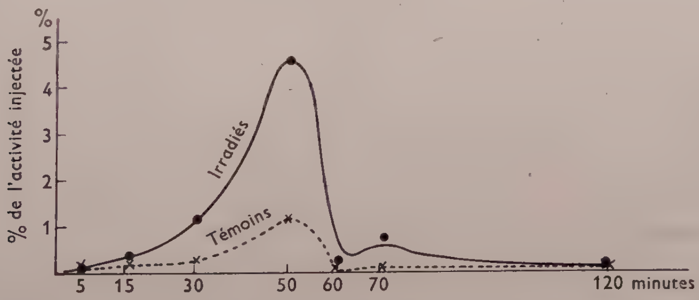


Fig. 3 b. Radioactivité (en p. 100 de l'activité injectée) du glycogène du foie de souris témoins et de souris irradiées avec 2000 r après l'injection de glucose marqué par du ^{14}C.

Rapport des aires entre 0 et 70 mn; $\frac{\text{Irradiés}}{\text{Témoins}} = 3$.

II. Résultats expérimentaux

Première série d'expériences.

Elle comprend 16 Irradiés et 16 Témoins. Les animaux ont été tués au bout d'un temps uniforme: 60 à 75 minutes, l'injection du glucose marqué a été faite soit immédiatement après l'irradiation soit au bout de 24 heures, et les doses de rayons ont été respectivement de 500 r, de 1000 r et de 2000 r.

Les résultats sont groupés dans le tableau suivant:

Doses d'irradiation	2 000 r			1 000 r	500 r	2 000 r[1]
		a		b	c	d
Durée de l'expérience	60′	70′	75′	70′	70′	70′
Rapport: Activité des Irradiés / Activité des Témoins						
Graisses	3,6	1,46		1,05	1,25	1,70
Glycogène . . .	1,64	2,63	2,78	0,96	0,94	1,4

Trois faits ont été mis en évidence:

1° Une irradiation de 2000 r augmente la quantité de ^{14}C retrouvée dans les graisses et le glycogène du foie 60 à 75 minutes après l'injection de glucose marqué (colonne a du tableau).

2° Elle l'augmente encore si l'injection est faite 24 heures après l'irradiation et non immédiatement après: l'effet est donc durable (colonne d du tableau).

3° Mais elle ne l'augmente plus ou seulement faiblement pour des doses de rayons plus faibles (colonne b et c du tableau).

La plus petite dose de rayons qui modifie d'une manière notable l'incorporation du ^{14}C dans les graisses et le glycogène du foie est donc 2000 r. C'est aussi cette dose de rayons qui, sur la souris, préserve le glycogène du foie pendant le jeune (4).

Deuxième série d'expériences.

Elle consiste en deux essais parallèles faits à plusieurs mois d'intervalle et comprenant chacun 36 Témoins et 36 Irradiés. Dans le deuxième essai seul les souris étaient de race pure (R. A. P. albinos) mais ce fait s'est par la suite révélé d'une importance mineure. La seule variable importante que nous ayons introduite entre les deux essais est la dose de rayons qui, de 2000 r dans le premier essai a été portée dans le deuxième à 2500 r.

Ces expériences ont été réalisées pour interpréter nos premiers résultats et rechercher si l'irradiation agit directement sur le turn-over des graisses et du glycogène, ou au contraire si ces constituants incorporent plus de ^{14}C parce qu'un autre métabolisme est perturbé par l'exposition aux rayons.

Dans ce dessein nous avons étudié la cinétique de l'incorporation du glucose marqué dans les graisses et le glycogène en tuant les animaux entre 5mn et 180mn après l'injection de glucose et en établissant les courbes représentant les variations d'activité en fonction du temps. Dans les figures 1 et 2 l'activité est exprimée en nombre d'impulsions par minutes; dans la figure 3, en p. 100 de l'activité injectée retrouvée dans la totalité des graisses et du glycogène.

---

[1] Dans cette expérience le glucose a été injecté 24 heures après l'irradiation.

Pour interpréter ces courbes nous avons examiné d'abord leur forme: pente, présence ou absence d'un plateau (12-13-14-15); nous avons ensuite comparé les surfaces limitées par ces courbes et l'axe des abscisses, surfaces dont nous avons calculé les valeurs relatives en pesant le papier sur lequel elles ont été dessinées et découpées. La comparaison, effectuée sur la figure 3, des témoins des deux séries a montré qu'ils incorporent dans la graisse et le glycogène exactement la même fraction du glucose injecté.

p. 100 de l'activité injectée retrouvée dans:

Les graisses Rapport: $\frac{\text{Témoin N° 1}}{\text{Témoin n° 2}} = 0.99$

Le glycogène Rapport: $\frac{\text{Témoin n° 1}}{\text{Témoin n° 2}} = 0.975$

Rapport $\frac{\text{Graisses}}{\text{Glycogène}}$ Témoin n° 1 4.75
Témoin n° 2 4.58

Bien que les races d'animaux et les conditions d'expériences soient différentes, les deux essais peuvent donc être comparés.

D'autre part, pour interpréter correctement nos courbes nous avons été amené à tenir compte de la vitesse avec laquelle le glucose injecté sous la peau passait dans la circulation. On sait en effet, que même en l'absence d'œdème et d'érythème, l'irradiation produit une vasodilatation superficielle due à la paralysie des sphincters précapillaires (16). Et l'on sait que, dans d'autres cas (17), ce phénomène s'accompagne d'une stase sanguine qui peut fort bien ralentir la résorption des solutions injectées sous la peau. C'est pourquoi nous avons été amené à mesurer la radioactivité du sang (figure 1 et 2, courbes a).

L'examen des figures 1 et 2 permet de dégager trois faits:

1° L'irradiation ralentit le passage dans le sang du glucose injecté sous la peau (courbes 1a et 2a).

2° Elle augmente la quantité de ^{14}C incorporé dans les graisses, notablement après 2500 r, faiblement après 2000 r (courbes 1b et 2b).

3° Elle augmente fortement la quantité de ^{14}C incorporé dans le glycogène (courbes 1c et 2c).

III. Interprétation et discussion

1° L'irradiation ralentit la résorption du glucose.

C'est cette interprétation que nous avons retenue pour expliquer les courbes 1a et 2a et non d'autres hypothèses auxquelles on aurait pu songer: augmentation de l'espace glucose, accélération de l'utilisation.

En effet, si l'espace glucose était augmenté le glucose ^{14}C serait dilué dans un plus grand volume de liquide et l'activité du sang serait à tout moment plus faible que chez les témoins: les courbes seraient parallèles et le resteraient tout au long de l'expérience. Il en serait encore ainsi si c'était l'utilisation du glucose qui était accélérée puisqu'à tout moment une grande quantité de glucose disparaîtrait de la circulation. Or ce n'est pas ce que l'on observe. Les courbes relatives aux Irradiés et aux Témoins se coupent, l'activité, d'abord plus faible chez les premiers devient ensuite plus forte et le reste jusqu'à la fin de l'expérience. En accord avec cette interprétation, les surfaces limitées par les deux courbes entre 0 et 70 mn sont semblables; leur rapport est de 1,01 (1a) et de 0,99 (2a). C'est donc la même quantité de glucose radioactif qui est passé dans la circulation entre 0 et 70 mn chez les Irradiés et chez les Témoins.

2° L'irradiation augmente la quantité de ^{14}C incorporé dans les graisses du foie.

Pour interpréter les faits relatifs aux graisses il est essentiel de distinguer les expériences de courtes durées, n'excédant pas 70 mn, de celles qui font intervenir un temps plus long.

Dans les expériences de courte durée comme le sont la majorité de celles qui sont reportées ici, la radioactivité des graisses est avant tout celle d'une fraction à métabolisme très rapide qui a été mise en évidence par HEVESY, RUYSSEN et BEECKMANS (18) et par BEECKMANS et de ELLIOT (19) sur des souris injectées avec de l'acétate marqué au carboxyle. Nos expériences montrent que, chez des animaux normaux, cette fraction incorpore le ^{14}C fourni par le glucose à peu près de la même manière que le ^{14}C provenant de l'acétate. L'activité des graisses est maxima 5 à 10 mm à peine après l'injection du glucose, puis elle décroit très rapidement pour croitre de nouveau et présenter entre 30 et 50 mm un deuxième clocher moins élevé que le premier (figures 1b et 2b, courbes des témoins).

On voit dans ces mêmes figures 1b et 2b, que l'irradiation augmente l'incorporation du ^{14}C dans cette fraction lipidique à métabolisme très rapide. La comparaison des aires limitées par les courbes des irradiés et des témoins met ce fait en évidence. du moins après une irradiation de 2500 r. Dans ce cas, bien que les irradiés résorbent le glucose plus lentement que les témoins, ils incorporent plus de ^{14}C dès le début de l'expérience. Le rapport des aires Irradiés/Témoins est toujours supérieur à l'unité: 1,57 lorsqu'on compare les activités spécifiques (Fig. 2b), 1.72 lorsqu'on compare, en p. 100 de l'activité injectée, l'activité retrouvée dans la totalité des graisses (Fig. 4a).

Après 2000 r le phénomène est beaucoup moins net, peut être parcequ'il est masqué par la lente résorption du glucose qui entraîne un retard notable dans l'incorporation du ^{14}C: le premier clocher est en effet déplacé jusque vers la vingtième minute. Les aires limitées par les courbes de la figure 1b, représentant les activités spécifiques, ne sont pas sensiblement différentes: leur rapport est de 1,08. Toutefois, lorsque l'activité est calculée en 100 de l'activité injectée retrouvée dans la totalité des graisses (Fig. 3a) le rapport est supérieur à l'unité: 1,21.

Dans les experiénces de longue durée, de plus de 70 mn (1b) la plus grande partie du ^{14}C se trouve dans la fraction lipidique à métabolisme plus lent. Il semble que dans cette partie de la courbe l'activité des graisses baisse moins vite chez les irradiés que chez les témoins, suggérant que l'irradiation diminue peut être aussi l'utilisation des graisses. Toutefois nos données expérimentales sont trop peu nombreuses pour que l'on puisse affirmer ce fait.

3° L'irradiation augmente fortement, de plus de trois fois, la quantité de ^{14}C incorporée dans le glycogène du foie. Le rapport des surfaces limitées par les courbes des irradiés et des témoins est de 3,35 après 2000 r et de 3,4 après 2500 r.

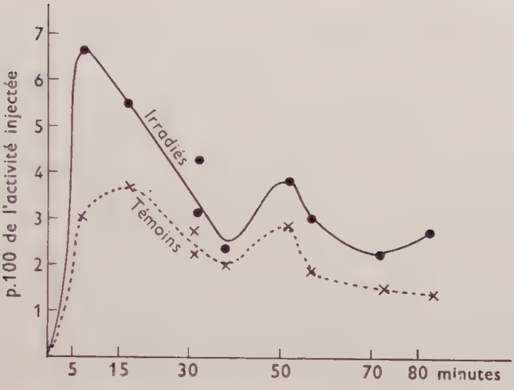


Fig. 4 a. Radioactivité (en p. 100 de l'activité injectée) des graisses extraites du foie de souris témoins et de souris irradiées avec 2500 r après l'injection de glucose marqué par du ^{14}C.

Rapport des aires entre 0 et 70 mn : $\frac{\text{Irradiés}}{\text{Témoins}} = 1,72$.

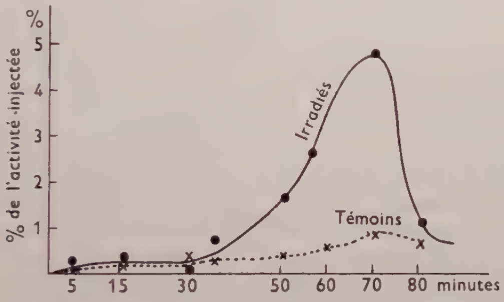


Fig. 4 b. Radioactivité (en p. 100 de l'activité injectée) du glycogène extrait du foie de souris témoins et de souris irradiées avec 2500 r après l'injection de glucose marqué par du ^{14}C.

Rapport des aires entre 0 et 70 mn : $\frac{\text{Irradiés}}{\text{Témoins}} = 3,45$.

Ce fait soulève un problème préliminaire, à savoir si l'activité de nos échantillons est celle du glycogène ou bien celle d'une impureté.

Nous avons écarté l'idée d'une impureté radioactive entraînée dans nos préparations; il ne peut y avoir de graisses puisque l'extraction est faite à partir de pulpe délipidée, et s'il y avait des bicarbonates ils donneraient de l'activité aux échantillons préparés dans les 30 minutes qui suivent l'injection, puisqu'ils sont eux mêmes actifs (9–10). Or ces échantillons n'ont jamais qu'une activité minime ou même pas d'activité du tout. L'incorporation du ^{14}C dans le glycogène ne commence réellement qu'au bout de 30 mn, et ceci confirme les données analytiques selon lesquelles le dépôt de glycogène dans le foie ne commence que 30 mm après l'ingestion du glucose (21).

Depuis que ce mémoire a été rédigé, l'auteur a verifié que le glycogène préparé dans les conditions de ces expériences ne contient pas d'impuretés radioactives en quantités appréciables.

En effet le glycogène purifié (par l'acide trichloracétique) est plus actif que le glycogène brut et les impuretés ne sont pas actives. D'autre part l'activité du glycogène purifié est intégralement conservée lorsque le glycogène, après avoir été hydrolysé, est transformé en glucosazone; en revanche l'activité disparaît totalement de la solution de glucose lorsque celui ci a été détruit par fermentation par la levure, et que l'on a éliminé l'alcool et le CO_2 qui ont été formés.

Par ailleurs, l'impureté principale qui pourrait subsister dans le glycogène purifié serait un ou des acides aminés. Or la ninhydrine n'en révèle que des traces (de l'ordre de 0,5 à 1 γ par 2 mg de glycogène) si faibles que nous n'avons pas pu les identifier par une chromatographie sur papier.

Mais une possibilité qui ne saurait être écartée est que la synthèse du glycogène se fasse en deux temps, comme celle des graisses, et que nos courbes représentent l'incorporation du ^{14}C non pas dans la masse principale du glycogène, mais dans un précurseur dont le renouvellement serait plus rapide.

Compte tenu de ces réserves nos expériences montrent que l'irradiation augmente l'incorporation du ^{14}C dans le glycogène du foie (ou dans un précurseur) ainsi que dans la fraction des graisses qui est très rapidement métabolisée.

Ce fait est en bon accord avec les données analytiques qui ont été rappellées au début de ce travail, à savoir que le glycogène est plus abondant chez des souris irradiées que chez des témoins jeunant depuis le même temps. Il est aussi en bon accord avec le fait bien connu que les graisses s'accumulent dans le foie des animaux irradiés, et que, chez beaucoup d'espèces, la stéatose est fonction de la dose de rayons reçue. C'est ainsi que chez le rat, la stéatose n'apparaît qu'après des doses léthales (22–23) et que chez la souris, elle est d'autant plus précoce que les doses de rayons sont élevées (ELLINGER 24). La technique des éléments marqués, qui est plus sensible que la technique histologique employée par Ellinger montre que chez la souris, il faut des doses très élevées, trois ou quatre fois mortelles, pour que le processus stéatosique débute tout de suite après l'irradiation. Ce n'est en effet qu'après 2500 r que l'activité spécifique des graisses est augmentée d'une manière certaine au premier point de nos courbes, c'est à dire 40 à 45 mm après le début de l'exposition aux rayons.

Comment peut on expliquer que l'irradiation augmente l'incorporation du ^{14}C fourni par le glucose?

Une première hypothèse, celle d'une baisse de la glycémie accompagnée d'une mise en réserve du glucose enlevé de la circulation, ne peut être retenue. En effet, le tableau ci joint montre que, conformément à ce que l'on observe chez le cobaye (25), la glycémie de la souris ne subit aucune variation pendant l'heure qui suit l'exposition aux rayons. Elle reste stable si les animaux sont nourris (partie 1 du tableau) et s'ils jeunent, elle ne baisse pas plus que chez des témoins soumis à un jeune de même durée (2ième partie du tableau).

Taux du sucre sanguin chez des souris témoins et chez des souris avant reçu une irradiation générale avec 2000 r.

I. Temps après l'irradiation	30 mn	40 mn	60 mn
Glycémies (en mg p. 100):			
Temoins	146	151	154
Irradiés	150	157	144

II. Temps après l'irradiation	0 mn	5 mn	15 mn	30 mn	45 mn	60 mn	70 mn
Glycémies (en mg p. 100):							
Témoins	130	127	117	111	87	87	79
Irradiés		125	122	98	89	83	81

Nous n'avons pas non plus retrouvé l'hyperglycémie constatée par THOMPSON et STEADMAN (26) sur le lapin.

Une deuxième explication est suggérée par les observations de HEVESY et FORSSBERG (6) et celles de HICKMAN et ASHWELL (27) qui tendent à montrer que l'irradiation diminue l'utilisation périphérique du glucose.

D'après HICKMAN et ASHWELL (27) la glycolyse anaérobie est diminuée dans des purées d'organes prélevés sur des animaux irradiés, et HEVESY et FORSSBERG (6) ont montré que le CO_2 exhalé par la souris est diminué, après l'irradiation, d'environ 20 p. 100. Or, une économie de 20 p. 100 sur le glucose métabolisé sous forme de CO_2 — qui représente lui-même 40 p. 100 du glucose total métabolisé (9-10) libère 8 p. 100 de glucose qui va être détourné vers d'autres métabolismes. Si ce glucose était tout entier transformé en glycogène, il porterait de 3 à 11 p. 100 la quantité de glucose utilisé dans cette synthèse, et cette augmentation serait en bon accord avec l'augmentation de l'activité spécifique du glycogène, qui, nous le rappellons, est d'environ trois fois et demi.

Mais dans nos expériences et en particulier dans la deuxième, l'activité spécifique des graisses augmente aussi et bien que cette augmentation soit plus modeste que celle du glycogène, elle n'en représente pas moins une quantité de glucose beaucoup plus considérable, la synthèse des graisses utilisant non plus 3 p. 100, mais 35 à 50 p. 100 du glucose total (9-10). Ainsi dans notre deuxième expérience, ce serait 17 à 25 p. 100 de glucose supplémentaire qui serait engagé dans la synthèse des graisses; cela veut dire qu'il existe d'autres sources de ^{14}C que celle qui provient de l'économie de glucose réalisée par la diminution de l'utilisation périphérique.

L'une de ces sources pourrait être le CO_2 lui-même puisqu'il est directement incorporé dans le glycogène (28–30–31). Toutefois, il faut remarquer que le CO_2 ne participe à cette synthèse d'une manière quelque peu marquée que chez des animaux à jeun qui viennent à être réalimentés. Chez des animaux normalement nourris comme l'étaient les nôtres, l'incorporation du CO_2 est toujours très faible (29) et ne peut guère entrer en ligne de compte dans le bilan de nos expériences.

Un autre mécanisme que l'on peut envisager est celui auquel ELY et ROSS (32–33) ont fait appel pour expliquer la haute teneur en glycogène des foies d'animaux irradiés et jeunant: l'augmentation du catabolisme protidique.

On sait en effet que le glycogène, comme les graisses, n'est pas synthétisé seulement à partir du glucose (ou des lipides alimentaires) mais aussi, même chez l'animal nourri, à partir de molécules plus petites qui sont fournies par un «fond» métabolique commun aux glucides, aux protides et aux lipides. L'augmentation de ce fond sous une influence quelquonque rompt l'équilibre dynamique en faveur de la mise en réserve et provoque l'incorporation dans les graisses et dans le glycogène de toutes les molécules qui constituent ce fond commun, quelque soit leur provenance.

Ce processus peut il intervenir dans nos expériences, est il possible qu'en l'espace de 45 mn l'irradiation libère des acides aminés, que ceux-ci soient désaminés et que le reste de la molécule soit engagé dans la synthèse des graisses et du glycogène?

Les données suivantes que nous avons trouvées dans la littérature sur la cinétique de ces diverses opérations ne permettent pas de rejeter cette possibilité.

1° L'augmentation du catabolisme protidique sous l'influence des rayons est importante et rapide: en 4 heures, les protéines structurales de l'intestin diminuent d'une manière quantitativement décelable (ELY et ROSS 32–33).

2° Les acides aminés sont très rapidement désaminés in vitro: WAELSCH (34) cite des expériences d'une durée de 60 mn, et KREBS (35) des expériences encore plus courtes: 20 mn et même 10 mn.

3° Enfin, le glycogène synthétisé par l'animal vivant à partir d'alanine (37–38) ou de cystéine et de cystine (39) forme des dépôts importants au bout de 3 heures et même de 2 heures.

Ces temps sont courts si l'on tient compte des techniques utilisées qui, étant toutes des techniques pondérables, ne mettent en évidence que des variations quantitatives importantes. En employant des éléments marqués, nous devons observer des modifications métaboliques bien avant que les variations pondérables puissent être révélées par une analyse quantitative.

Résumé.

1° Des souris ont reçu une injection de glucose uniformément marqué par du ^{14}C immédiatement après avoir été exposées à une irradiation générale par les rayons X, aux doses de 2000 r et 2500 r.

2° L'irradiation ralentit la résorption du glucose injecté sous la peau.

3° L'irradiation augmente la quantité de ^{14}C incorporé dans la fraction des lipides hépatiques dont le métabolisme est très rapide. L'augmentation est importante après une dose de 2500 r, mais peu marquée ou même douteuse après 2000 r.

4° L'irradiation augmente considérablement, de plus de trois fois, la quantité de ^{14}C incorporé dans le glycogène du foie, ou dans un précurseur.

5° Les mécanismes qui ont été envisagés pour expliquer ces faits sont discutés.

L'auteur désire exprimer ses remerciements à Mr le Professeur G. HEVESY qui lui a permis de réaliser ce travail et qui a bien voulu en discuter les résultats et l'interprétation.

L'auteur remercie également le Dr A. FORSSBERG (Karolinska Sjukhuset) et le Dr. F. BACLESSE (Foundation Curie) d'avoir bien voulu irradier les animaux, et MMelles G. DREYFUSS, R. PETZOLD, et E. ESTIENNE de l'avoir aidée dans ces expériences.

BIBLIOGRAPHIE

1. NORTH, N. and NIMS, L. F., *Fed. Proc.* 1949, *8*, 119.
   NIMS, L. F., *J. Chim. Physique* 1951, *48*, 280.
2. ROSS, M. H. and ELY, J. O., *J. cell. comp. Physiol.* 1951, *37*, 163.
3. STOCKEN, L. A., *Chemistry and Industry* 1951, *no. 52*, 1166.
4. LOURAU, M., *C. R. Ac. Sc.* 1953, *236*, 422.
5. ROSS, M. H. and ELY, J. O., *Am. J. Roentgen. Rad. Therap.* 1949, *62*, 723.
6. HEVESY, G. and FORSSBERG, A., *Nature* 1951, *168*, 692.
7. DUBOIS, K. P., COCHRAN, K. W. and DOULL, J., *Proc. Soc. Exp. Biol. Med.* 1951, *76*, 422.
8. DOUNCE, A. L., *J. Cell. Comp. Physiol.* 1952, *39*, Supp. 2, 43.
9. FELLER, D. D., STRISOWER, E. H. and CHAÏKOFF, I. L., *J. Biol. Chem.* 1950, *187*, 589.
10. DRURY, D. R., WICK, A. N., BANCROFT, R. W. and MAC KAY, E. M., *Am. J. Physiol.* 1951, *164*, 207.
11. STETTEN, W. and BOXER, G. E., *J. Biol. Chem.* 1944, *155*, 231 et 237.
12. HEVESY, G., *Nature* 1949, *164*, 1007.
13. HEVESY, G., RUYSSEN, R. et BEECKMANS, M. L., *Experientia* 1951, *7*, 317.
14. BEECKMANS, M. L., CASIER, H. et HEVESY, G., *Arch. Int. Pharmacodyn.* 1951, *86*, 33.
15. HEVESY, G. and DREYFUSS, G., *Arkiv för Kemi* 1952, *4*, 337.
16. MAC CUTCHEON, M., *J. Cell. Comp. Physiol.* 1952, *39*, 113.
17. CHAMBERS, R. and ZWEIFACH, B. W., *Physiol. Rev.* 1947, *27*, 436.
18. HEVESY, G., RUYSSEN, R., and BEECKMANS, M. L. *Experientia* 1951, *7*, 144.

19. BEECKMANS, M. L. et de ELLIOTT, G., *Nature* 1951, *167*, 200.
20. BUCHANAN, J. M., HASTINGS, A. B. and NESBETT, F. B., *J. Biol. Chem.* 1943, *150*, 413.
21. LOURAU, M. et LARTIGUE, O., *J. de Physiol.* 1951, *53*, 593.
22. CONIGLIO, J. G., DARBY, W. J., WILKERSON, M. C., STEWART, R., STOCKELL, A. and HUDSON, G., *Amer. J. Physiol.* 1953, *172*, 86.
23. LEBLOND, C. P., THOAI, N. et SEGAL, *C. R. Soc. Biol.* 1939, *130*, 1557.
24. ELLINGER, F. R., *Radiology* 1945, *44*, 241.
25. ROTHER, J., *Strahlentheraphie* 1928, *27*, 197.
26. THOMPSON, H. E. and STEADMAN, L. T., *Nuclear Science Abstracts* 1951, *5*, 607.
27. HICKMAN, J. and ASHWELL, G., *Fed. Proc.* 1953, *12*, 66.
28. VENNESLAND, B., SOLOMON, A. K., BUCHANAN, J. M. and HASTINGS, A. B., *J. Biol. Chem.* 1942, *142*, 379.
29. SOLOMON, A. K., VENNESLAND, B., KLEMPERER, F. W., BUCHANAN, J. M. and HASTINGS, A. B., *J. Biol. Chem.* 1941, *140*, 171.
30. WOOD, H. G., *Cold Spring Harbor Symposia on quantitative Biology* 1948, *13*, 201.
31. BUCHANAN, J. M. and HASTINGS, A. B., *Physiological Reviews* 1946, *26*, 198.
32. ELY, J. O. and ROSS, M. H., *Cancer Research* 1948, *8*, 285.
33. ROSS, M. H. and ELY, J. O., *Amer. J. Roentgenol.* 1949, *62*, 718.
34. WAELSCH, H., *Adv. in Enzymol.* Vol. *13*, 237.
35. KREBS, H. A., *Bioch. J.* 1935, *29*, 1620.
36. WILSON, R. H. and LEWIS, H., *J. Biol. Chem.* 1929, *84*, 511.
37. MAC KAY, E. M., WICK, A. N. and BARNUM, C. P., *J. Biol. Chem.* 1941, *137*, 183.
38. SCHOFIELD, F. A. and LEWIS, H. B., *J. Biol. Chem.* 1947, *169*, 373.
39. HESS, W. C., *J. Biol. Chem.* 1949, *181*, 23.

Institut de Chimie Biologique et Organique de l'Université de Stockholm.

Tryckt den 13 maj 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 25

Communicated 10 March 1954 by KARL MYRBÄCK

Some derivatives of 2-amino-4-ethylpyridine

By KJELL HALVARSON

For some experiments on nucleophilic aromatic substitution the following substances were prepared: 2-amino-3,5-dibromo-4-ethylpyridine, 4-ethyl-2-nitraminopyridine, 4-ethyl-2-hydrazinopyridine, and benzaldehyde 4-ethyl-2-pyridylhydrazone. The methods involved have been used previously by other investigators. The starting material was 4-ethylpyridine, which was prepared according to the directions given in Organic Syntheses (1).

Experimental

2-amino-4-ethylpyridine has been prepared earlier (2), but the method now used follows the general scheme given in Organic Reactions (3), which is useful for the preparation of small amounts of aminopyridines. The directions given in detail were followed in almost every respect, but the amount of material used was $\frac{1}{3}$ to $\frac{1}{2}$ of that used in the description. The amination temperature was 115–120° C, the higher temperature being used for starting the reaction, which will then proceed even if the temperature falls to 110–112° C. The 2-amino-4-ethylpyridine was distilled (125–128° C, 13 mm) from the reaction mixture, instead of being extracted with benzene. Crystallized from ligroin and decolorized with animal charcoal it gives white flakes with m.p. 66.5–7.5° C. The yield in three different syntheses was 52–64 %.

2-amino-3,5-dibromo-4-ethylpyridine. 1.0 g of 2-amino-4-ethylpyridine was added to 3.0 ml of 20 % sulphuric acid, and 0.85 ml of bromine then added slowly. The mixture was heated on a water bath for about four hours and then placed in a refrigerator (about 0° C), where it was left overnight. The crystal mass was sucked off and washed with some cold 20 % sulphuric acid, and then with water. After drying and two recrystallizations from ligroin, including treatment with animal charcoal, it melts at 113.5–114.0° C. Bright white plates. Yield 82 % of crude material and 65 % of crystallized material.

$C_7H_8N_2Br_2$[1]	C	H	N	Br
% calc.	30.03	2.88	10.01	57.09
% found	29.95	2.88	10.05	56.94

---

[1] The author is indebted to Mrs. MAJBRITT VESTERGAARD, who carried out the analyses.

4-ethyl-2-nitraminopyridine was prepared in the same manner as SEIDE (4) prepared 4-methyl-2-nitraminopyridine.

6.7 g of 2-amino-4-ethylpyridine was dissolved in 55 ml of concentrated sulphuric acid cooled with ice and salt, and then 5.85 g of nitric acid (65 %, d = 1.4) was added slowly by drops with frequent stirring. The mixture was stirred for about one hour at a temperature of about 0° C. The reaction mixture was then poured onto ice and the white crystal mass sucked off and washed with water. Crystallization from alcohol gives white crystals which turn yellowish after some time. The substance darkens at about 160° and melts during decomposition at 171–2° C. Yield 88 %.

$C_7H_9O_2N_3$	C	H	N
% calc.	50.29	5.43	25.14
% found	50.07	5.48	25.20

4-ethyl-2-hydrazinopyridine. CHICHIBABIN and RAZORENOV (5) have prepared 2-hydrazinopyridine, and the 4-ethyl-2-hydrazinopyridine was prepared in about the same manner.

4.5 g of 4-ethyl-2-nitraminopyridine was added to 90 ml of 10 % ice-cooled sodium hydroxide and 7 g of zinc powder was added to the stirred solution. The mixture was left overnight in a refrigerator (about 0° C, with continuous stirring). The oil obtained, part of which floated on the surface, was extracted with a little benzene and the benzene extract dried with solid sodium hydroxide. The filtered solution was distilled off under reduced pressure in a nitrogen atmosphere, and the material boiling between 138–142° C at 10 mm pressure was collected. This substance crystallized at about 0° C, but contained about 10 % of 2-amino-4-ethylpyridine. For purification, the crystals were dissolved in petrol ether, and recrystallized with cooling in a dry-ice bath. The white crystal mass was filtered off with suction and dried in a desiccator in an atmosphere of some millimeters of nitrogen. The substance melts at 19–22° C and the yield of the crude product is 52 % and of crystallized material about 30 %.

$C_7H_{11}N_3$	C	H	N
% calc.	61.28	8.08	30.64
% found	61.27	8.03	30.07

If the reduction is carried out at room temperature more 2-amino-4-ethylpyridine is obtained as a by-product. Sodium amalgam may be used instead of zinc and sodium hydroxide in the reduction, but the method described above seems to be the better one.

Benzaldehyde 4-ethyl-2-pyridylhydrazone. In order to get a stable compound for the characterization of the hydrazine derivative, the corresponding hydrazone of benzaldehyde was prepared. The two compounds were dissolved in ethanol and the hydrazone precipitated by the addition of some water. The compound was recrystallized from slightly aqueous methanol. White needles with m.p. 149.0–149.5° C. (Found: N 18.71 %. Calc. for $C_{14}H_{15}N_3$: N 18.66 %).

All temperatures given are uncorrected.

Nobel Institute of Chemistry, Stockholm 50, Sweden.

REFERENCES

1. Organic Syntheses, Vol. 27, New York (1948), p. 38.
2. Chem. Fabr. Schering, Horsters, Dohrn, Ger.pat. 398204 (1924).
3. Organic Reactions, Vol. I, New York (1947), p. 91.
4. SEIDE, O., Ber. *57*, 794 (1924).
5. CHICHIBABIN, A. E., and RAZORENOV, B. A., J. Russ. Phys. Chem. Soc. *47*, 1286 (1915).

Tryckt den 22 maj 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

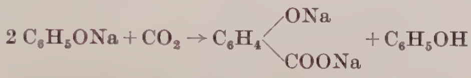
ARKIV FÖR KEMI Band 7 nr 26

Communicated 10 March 1954 by ARNE FREDGA and GUNNAR HÄGG

Some aspects of Kolbe's synthesis of salicylic acid

By SIGVARD WIDEQVIST

According to the well-known synthesis of KOLBE [1] salicylic acid is prepared by heating sodium phenoxide together with carbon dioxide under pressure at 180–200°. In this way half the quantity of the phenoxide is converted to the disodium salt of salicylic acid:



$$2\,C_6H_5ONa + CO_2 \rightarrow C_6H_4\begin{matrix}ONa\\COONa\end{matrix} + C_6H_5OH$$

It was previously proposed that the first stage of this process involved the formation of phenyl sodium carbonate, $C_6H_5OCOONa$, which then rearranged, since this is the first product which can be isolated from the reaction. It is, however, likely that the initial appearance of phenyl sodium carbonate has little or no importance for the formation of salicylic acid, as it probably dissociates again into sodium phenoxide and carbon dioxide at elevated temperature. In fact, it has been shown by SILIN and MOSHCHINSKAYA [2] that when sodium 2-naphthoxide is carbonized at 40–60°, the first product is 2-naphthyl sodium carbonate. At 120° the reversible reaction

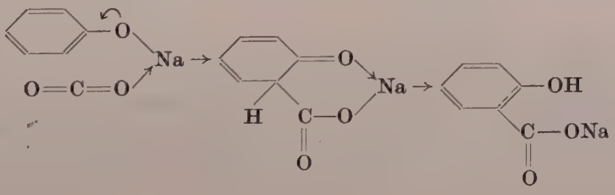
$$2\text{-}C_{10}H_7OCO_2Na \rightleftharpoons 2\text{-}C_{10}H_7ONa + CO_2$$

is shifted to the right, and 2-hydroxy-1-naphthoic acid is formed at 120–145°; at 280–290° the product is 3-hydroxy-2-naphthoic acid (SCHMITT and BURKARD [3]).

However, when potassium phenoxide is substituted for sodium phenoxide in the Kolbe reaction, the product is, curiously enough, not salicylic acid but *p*-hydroxybenzoic acid (KOLBE [4]).

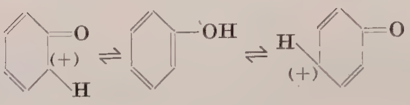
It is, however, to be noticed that the *ortho*-derivative is the first reaction product also when potassium phenoxide is used; on further heating it is converted into the *para*-product. As found by OST [5], potassium salicylate is actually converted into the dipotassium salt of *p*-hydroxybenzoic acid on heating at 230°. This method gives excellent yields of *p*-hydroxybenzoic acid (*Org. Synth.* [6]).

According to JOHNSON [7] the formation of salicylic acid from sodium phenoxide takes place through a metal chelate compound:



but no explanation is given for the isomerization of potassium salicylate to potassium *p*-hydroxybenzoate.

WALTER HÜCKEL [8] assumes that the phenol reacts in a chinolidic form

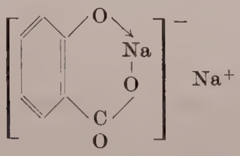


which adds the carbon dioxide directly. He says that it seems mysterious that the *p*-chinolidic form is preferred when the potassium salt is used.

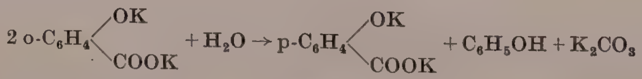
FUSON [9], however, says as follows with reference to the isomerization of 2-hydroxy-1-naphthoic acid into 3-hydroxy-2-naphthoic acid: "The result suggests that the Kolbe reaction is reversible, that the carboxyl group enters the *alpha* position *readily but reversibly*, whereas its introduction into the 3 position, though difficult, is *essentially irreversible. A similar explanation may be advanced for the isomerization of potassium salicylate to potassium p-hydroxybenzoate*". (The italics are those of the present author.)

It should, however, be mentioned that the formation of *p*-hydroxybenzoic acid is evidently reversible also. KUPFERBERG [10] actually found that *p*-hydroxybenzoic acid, as sodium salt, on heating, is partially reconverted into salicylic acid.

It is thus evident that there is some property of the sodium salt which is responsible for the preservation of the salicylic acid structure. It seems possible that an at least contributory cause would be the stability of the chelate compound, which was previously mentioned as an intermediate product in the synthesis of salicylic acid. It might also, and this is far more likely, depend on the stability of the disodium salt, to which also a chelate structure can be attributed:



There is a considerable difference between the stabilities of the secondary sodium and potassium salicylates. Thus, disodium salicylate can be heated to near 300° without any decomposition. Dipotassium salicylate, however, is completely decomposed into dipotassium *p*-hydroxybenzoate, phenol, and potassium carbonate on heating to 220°, as found by OST. The reaction formula given by him is somewhat curious, as it requires the presence of water:



$$2\ \text{o-}C_6H_4\left\langle\begin{matrix}OK\\COOK\end{matrix}\right. + H_2O \rightarrow \text{p-}C_6H_4\left\langle\begin{matrix}OK\\COOK\end{matrix}\right. + C_6H_5OH + K_2CO_3$$

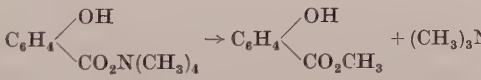
and it can be questioned, where the water comes from. OST assumed that the dipotassium salicylate contained some water, which could not be removed by drying. The potassium salt is indeed very hygroscopic.

According to MARTELL and CALVIN [11] the tendency for chelate formation is much greater for sodium than for potassium. It has further been demonstrated that the tendency for chelate formation increases with increasing charge-radius ratio for the chelating metal ion. It would thus be expected that rubidium and cesium salicylate would, like potassium salicylate, give *p*-hydroxybenzoic acid on heating. The lithium salt, however, would not be expected to rearrange. This in fact has been shown experimentally. As found by VON DEN VELDEN [12], rubidium salicylate is converted into dirubidium *p*-hydroxybenzoate when heated at 250°. The present author found that cesium salicylate reacts in the same way. VON DEN VELDEN also found that lithium salicylate is not rearranged to *p*-hydroxybenzoate.

The conception of the greater chelating tendency of the sodium salicylate is also consistent with the results obtained on measurements of the electrical conductivity of alkali salicylates in a non-hydroxylic solvent. DIPPY, JENKINS, and PAGE [13] thus found that the conductivities of sodium and potassium salicylate in acetone were surprisingly low and that the ionization constant of sodium salicylate was about a tenth of that of potassium salicylate. The ionization constants, calculated according to Ostwald's dilution formula, were $2.76 \cdot 10^{-5}$ and $2.0 \cdot 10^{-4}$, respectively. It should, however, be mentioned that the limiting conductance Λ_0, used by DIPPY, JENKINS, and PAGE, was only a guess, and their "constants" were strongly depending on the concentration.

In order to find out if there were any great differences between the dissociation constants of the other alkali salicylates, the present author studied also the conductivities of these salts in acetone. It was found that the dissociation constants of the potassium, rubidium, and cesium salicylates were of the same order of magnitude but increased somewhat with the ion radius of the alkali metal, as might be expected. The lithium salicylate, however, was a much poorer conductor.

Conductivity measurements were also performed on tetramethylammonium salicylate. The behaviour of the tetramethylammonium salt on heating was also investigated. It decomposed completely with the production of trimethylamine and methyl salicylate:



$$C_6H_4\begin{cases}OH\\CO_2N(CH_3)_4\end{cases} \rightarrow C_6H_4\begin{cases}OH\\CO_2CH_3\end{cases} + (CH_3)_3N.$$

Experimental

The alkali salicylates were prepared by neutralizing salicylic acid with the appropriate carbonates. A slight excess of salicylic acid was used. The solutions were evaporated to dryness and the residues dried in vacuo over sulfuric acid. The salts were then dissolved in dry acetone or methanol, and ether was carefully added, causing the salts to separate. Potassium salicylate was recrystallized from hot ethyl alcohol. The salts were kept in a vacuum desiccator over phosphorous pentoxide.

Tetramethylammonium salicylate was prepared by neutralizing salicylic acid with tetramethylammonium ethoxide: Sodium, 2.3 g, was dissolved in 25 ml of absolute alcohol, and the solution was added to a solution of 5.48 g of tetramethylammonium chloride in 25 ml of boiling absolute alcohol. After cooling, the separated sodium chloride was filtered off, and 7.0 g of salicylic acid was added. The solution was filtered, and ether was carefully added, causing the tetramethylammonium salicylate to separate as glistening crystals. Yield 7 g. The salt was filtered off, thoroughly washed with ether and dried in vacuo over sulfuric acid. It was then recrystallized from acetone and ether. Tetramethylammonium salicylate is very hygroscopic and liquefies rapidly when kept in the air. On heating it decomposes yielding trimethylamine and methyl salicylate.

All the salts are easily soluble in acetone, alcohol and methanol. It seems, however, that the potassium salt is less soluble than the other alkali salts in acetone.

The pyrolysis of cesium salicylate. In a small test tube 0.54 g (0.02 mole) of cesium salicylate was heated at 240° in a metal bath, until the evolution of carbon dioxide entirely ceased. This required about half an hour. The residue was dissolved in water, giving a deep red solution. The solution was boiled with some decolorizing charcoal and filtered hot. The light red filtrate was acidified with concd. hydrochloric acid, and the color turned to yellow. The solution was completely decolorized when treated with a fresh quantity of charcoal. When the solution was concentrated, white crystals separated. They were filtered off and recrystallized from a small quantity of water. The final product was dried at 100°. M.P. 214°. When mixed with an authentic sample of *p*-hydroxybenzoic acid, the product showed the same melting point.

The acetone used in the conductivity measurements was "AnalaR" from Hopkin and Williams Ltd. The same product was also used by DIPPY, JENKINS, and PAGE. They purified the acetone by treating it with anhydrous calcium chloride and a small quantity of potassium carbonate. In the present investigation it was preferred to dry the acetone at first over anhydrous potassium carbonate and then over Drierite (anh. $CaSO_4$). The latter drying agent must be more efficient than calcium chloride, because of the extremely low equilibrium vapor pressure over the system

$$2\,CaSO_4 + H_2O \rightleftharpoons 2\,CaSO_4,\ H_2O$$

which amounts to only 0.004 mm/Hg at 25°. After drying the acetone was distilled through a Widmer column and protected against moisture as far as possible. The specific conductivity of the acetone was $0.094 - 0.140 \cdot 10^{-6}$.

The conductivity measurements were performed at 25° C. The solutions of the alkali salicylates were prepared at room temperature and the volumes of the solutions were corrected to 25°. It was assumed that the density of the solutions was the same as that of the pure solvent. The density of acetone at different temperatures was calculated according to the formula

$$d\,(\mathrm{g/cc}) = 1.091301 - 92.722 \cdot 10^{-5}\,T - 32.331 \cdot 10^{-8}\,T^2$$

given by FELSING and DURBAN [15].

The constants in Onsager's equation (Eq. (11)) for acetone were taken from DAVIES [16] and had the values $a = 1.627$ and $b = 326.7$. For the dielectric constant of acetone very different values are recorded in the literature. It was, however, considered to be most correct to take also this quantity from DAVIES, who gives $D = 21.2$.

Methods of calculation

All calculations of the dissociation constant of an electrolyte from conductivity measurements involve the determination of the limiting conductivity, Λ_0, at infinite dilution. In the case of weak acids in aqueous solution, Λ_0 is usually calculated from measurements on their alkali salts, which are assumed to be completely dissociated. The relation between Λ_0 and the equivalent conductivity Λ_c at the concentration c for a strong electrolyte is given by Onsager's equation at low concentrations:

$$\Lambda_c = \Lambda_0 - (a\,\Lambda_0 + b)\sqrt{c} \tag{1}$$

where a and b are parameters characteristic for the solvent. In solvents with low dielectric constant, however, salts are also highly associated even at low concentrations. The salts then behave as weak electrolytes, and a dissociation constant may be calculated. It is, however, necessary in this case to determine both the limiting conductivity and the dissociation constant from the same data, and several methods are available for this purpose. For less accurate calculations the formula of KRAUS and BRAY [17]

$$\frac{1}{\Lambda_c} = \frac{1}{\Lambda_0} + \frac{c\,\Lambda_c}{K\,\Lambda_0^2} \tag{2}$$

may be used. This equation is based upon Ostwald's dilution law:

$$\frac{c\,\Lambda_c^2}{\Lambda_0\,(\Lambda_0 - \Lambda_c)} = K. \tag{3}$$

If $1/\Lambda_c$ is plotted against $c\,\Lambda_c$, then $1/\Lambda_0$ and $1/(K\,\Lambda_0^2)$ are determined as intercept and slope, respectively.

More exact methods which take account of the ion activities and the dependence of the ion mobilities upon the ion concentration have been worked out by IVES [18], FUOSS and KRAUS [19], FUOSS [14], and SHEDLOVSKY [20]. In this paper the method of FUOSS was used for the calculation of the thermodynamic dissociation constants.

For a completely dissociated electrolyte, the conductance would be $\Lambda_0 - (a\,\Lambda_0 + b)\sqrt{c}$ at high dilution (Eq. (1)). If the degree of ionization of the electrolyte is α, then the ion concentration is αc, and the equivalent conductance would be

$$\Lambda_c = \alpha\,[\Lambda_0 - (a\,\Lambda_0 + b)\sqrt{\alpha c}] \tag{4}$$

and thus the degree of ionization

$$\alpha = \frac{\Lambda_c}{\Lambda_0\,[1 - (a\,\Lambda_0 + b)\sqrt{\alpha c}/\Lambda_0]}. \tag{5}$$

In the limit, eq. (5) reduces to $\alpha = \Lambda_c/\Lambda_0$, upon which Ostwald's dilution formula is based. By means of successive substitution of eq. (5) back into its own correction term, $1 - (a\,\Lambda_0 + b)\sqrt{\alpha c}/\Lambda_0$, the latter may be replaced by the continued fraction

$$F(Z) = 1 - Z\{1 - Z[1 - Z\,(\text{etc.})^{-\frac{1}{2}}]^{-\frac{1}{2}}\}^{-\frac{1}{2}} \tag{6}$$

in which

$$Z = (a\,\Lambda_0 + b)\,\Lambda_0^{-\frac{3}{2}}\sqrt{\Lambda_c\,c}. \tag{7}$$

Fuoss [14] tabulated numerical values of $F(Z)$ for $0 \leq Z \leq 0.209$ by intervals of 0.001, so that α can be calculated by

$$\alpha = \frac{\Lambda_c}{\Lambda_0\,F(Z)} = \frac{\Lambda_c}{\Lambda_x} \tag{8}$$

when Λ_0 is known. The evaluation of Λ_0 requires a short series of successive approximation, which may begin with a rough value of Λ_0 obtained by guess or by the formula of Kraus and Bray (Eq. (2)). This preliminary value of Λ_0 leads to approximate values of $F(Z)$ and α. The values of α are used to estimate the activity coefficients by the limiting law

$$\log f = -A\sqrt{\alpha\,c}. \tag{9}$$

The coefficient A is calculated from the equation

$$A = 1.815 \cdot 10^6/(DT)^{\frac{3}{2}} \tag{10}$$

where D is the dielectric constant of the solution.

When eq. (8) is combined with the expression for the thermodynamic dissociation constant

$$K_0 = \frac{f^2\,\alpha^2\,c}{1-\alpha} = f^2\,K_c \tag{11}$$

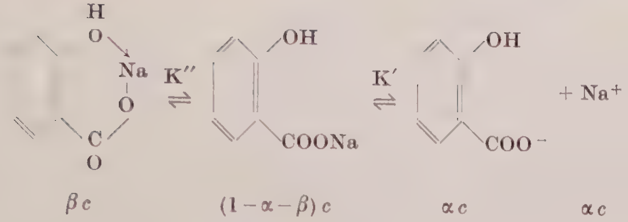
we obtain according to Fuoss the equation

$$\frac{F(Z)}{\Lambda_c} = \frac{1}{\Lambda_0} + \frac{[c\,\Lambda_c\,f^2/F(Z)]}{K_0\,\Lambda_0^2} \tag{12}$$

which at infinite dilution reduces to the formula of Kraus and Bray (Eq. (2)). A plot of $F(Z)/\Lambda_c$ against $c\,\Lambda_c\,f^2/F(Z)$ gives $1/(K_0\,\Lambda_0^2)$ as the slope and $1/\Lambda_0$ as the intercept. The Λ_0 obtained in this way is usually not identical with the correct value but may be used to construct a new plot, using eqs. (7), (9), (12), from which a more accurate value of Λ_0 is obtained. This process is repeated until a final value of Λ_0 is found to satisfy all the equations (7), (9), (12). Eq. (12) is best treated by the method of least squares.

It can be discussed, what meaning the dissociation constant has for an alkali salicylate in acetone. Can it be assumed that the salt exists only in ions and chelated molecules, or must it be taken into account that there are also associated ion pairs?

Let us assume that the fraction of the electrolyte present as ions is α and that present as chelate is β and the equilibrium constants are K' and K'', respectively:



Then we get

$$\frac{\alpha^2 f^2 c}{1-\alpha-\beta} = K' \tag{13}$$

$$\beta/(1-\alpha-\beta) = K''. \tag{14}$$

From eq. (14) we get

$$\beta = K''(1-\alpha)/(1+K''). \tag{15}$$

When the value of β is introduced into eq. (13), we obtain

$$\frac{\alpha^2 f^2 c}{1-\alpha-\dfrac{K''(1-\alpha)}{1+K''}} = K'$$

and after simplification

$$\frac{\alpha^2 f^2 c}{1-\alpha} = \frac{K'}{1+K''} = K_0. \tag{16}$$

As seen from eq. (16), the left hand side acquires a constant value, identical with K_0, even if there is an equilibrium between chelated molecules and associated ion pairs. If K' and K'' could be determined separately, it would be possible to calculate the heat of dissociation of the chelate bond and thus get a measure of the tendency of the chelate formation.

Results of measurements and discussion

The dissociation constants of sodium and potassium salicylate obtained by Dippy, Jenkins, and Page [13] were calculated by means of Ostwald's dilution formula. The limiting conductance, Λ_0, was, however, rather arbitrarily chosen, and their dissociation "constants" were therefore in no way constant. The dissociation constant of sodium salicylate thus increased from $2.33 \cdot 10^{-5}$ to $3.02 \cdot 10^{-5}$ in the concentration interval $9.448 - 22.40 \cdot 10^{-5}$. The mean value was $2.76 \cdot 10^{-5}$. For potassium salicylate the mean value $2.0 \cdot 10^{-4}$ was obtained. An inspection of the experimental data showed that those of potassium salicylate were much better than those of sodium salicylate. This is, however, not surprising, as it must be much more difficult to perform accurate measurements on the slightly dissociated sodium salt. In order to get a more reliable comparison between the dissociation constants of the sodium and potassium salts an attempt was made to calculate thermodynamic dissociation constants by the method of FUOSS [14] from the data of DIPPY, JENKINS, and PAGE. As it was desirable to determine the limiting conductance of the salicylate ion as accurate as possible, measurements were also performed on the relatively highly dissociated tetramethylammonium salicylate. From these measurements the limiting conductance 189.1 for tetramethylammonium salicylate was obtained. According to WALDEN, ULICH, and BUSCH [21] the mobility of the tetramethylammonium ion in acetone is 102.8. From this the mobility 86.3 is obtained for the salicylate ion. According to the same authors the mobilities of the potassium ion and the sodium ion are 69.7 and 68.6, respectively. Thus we obtain the limiting conductivity 156.0 for potassium salicylate. In good agreement with this the value 155.6 is calculated according to FUOSS from the data of DIPPY, JENKINS, and PAGE.

The measurements on rubidium and cesium salicylate were treated by the formula of KRAUS and BRAY (Eq. (2)). It was not considered to be correct to extrapolate thermodynamic dissociation constants from the data, as they were not accurate enough. The apparatus used for the measurements was unfortunately not suitable for measurements in media of low dielectric constants. The measurements could therefore not be extended to as high dilution as would be necessary to make a reliable extrapolation to the limiting conductance Λ_0. Nor was it advisable to calculate Λ_0, as the ion mobilities in acetone for rubidium and cesium, available in the literature, seem questionable. LANNUNG [22] thus records $\Lambda_{\mathrm{Rb}} = 86$ and $\Lambda_{\mathrm{Cs}} = 88$ (from the measurements by N. L. ROSS KANE). But according to the same author $\Lambda_{\mathrm{K}} = 82$, when WALDEN, ULICH, and BUSCH [21] give $\Lambda_{\mathrm{K}} = 69.7$. Because of this discrepancy it might be suspected that the values of Λ_{Rb} and Λ_{Cs} are less accurate. The dissociation constants of rubidium and cesium salicylate are thus to be regarded as somewhat approximate.

In order to get a comparison between all the alkali salicylates classical dissociation constants were also calculated for sodium and potassium salicylate. The results are recorded in the Tables 1–5.

In the tables the symbols have the following meaning:

C total concentration of the electrolyte,
C_i ion concentration,
Λ_0 equivalent conductance at infinite dilution,
Λ_0^* limiting conductance from the formula of KRAUS and BRAY,
α^* degree of dissociation, calculated under the assumption of constant ion mobility (Λ_0^*),
K_0 thermodynamic dissociation constant,
K classical dissociation constant,
Λ_c equivalent conductance at concentration C,
Λ_x $F(Z)\,\Lambda_0$.

It should be mentioned that the limiting conductance $\Lambda_0 = 154.9$ for sodium salicylate is calculated from the ion mobilities and not from the conductivity measurements on the sodium salt.

Lithium salicylate was a poorer conductor than the other alkali salicylates. It was thus necessary to perform the measurements at relatively high concentra-

Table 1. Dissociation constant of tetramethylammonium salicylate.

$\Lambda_0 = 189.1.$

$C \cdot 10^3$	Λ_c	Λ_x	$C_i \cdot 10^3$	$K_0 \cdot 10^3$
0.5435	144.8	175.7	0.4480	1.482
0.8207	134.5	173.1	0.6379	1.467
1.081	127.7	171.1	0.8069	1.487
1.338	121.8	169.4	0.9620	1.476
2.046	110.5	165.7	1.365	1.486
2.701	102.9	162.9	1.706	1.480
3.102	99.16	161.4	1.906	1.477
4.032	92.33	158.5	2.348	1.472

Mean value $K_0 = 1.478 \cdot 10^{-3}$

Table 2. Dissociation constant of sodium salicylate.

$\Lambda_0 = 154.9;\ \Lambda_0^* = 143.9.$

(From the data of DIPPY, JENKINS, and PAGE.)

$C \cdot 10^4$	Λ_c	Λ_x	$C_i \cdot 10^4$	α^*	$K_0 \cdot 10^5$	$K \cdot 10^5$
0.2810	98.91	152.4	0.1823	0.6874	3.14	4.25
0.3497	94.32	152.2	0.2167	0.6555	3.27	4.36
0.4041	93.78	152.0	0.2493	0.6517	3.70	4.93
0.4149	90.59	152.0	0.2473	0.6295	3.36	4.44
0.5299	84.72	151.8	0.2958	0.5887	3.42	4.47
0.5988	83.52	151.6	0.3299	0.5804	3.68	4.81
0.6905	78.20	151.4	0.3566	0.5434	3.45	4.47
0.6966	75.41	151.5	0.3468	0.5240	3.12	4.02
0.9448	66.01	151.2	0.4126	0.4587	2.88	3.67
1.277	63.53	150.7	0.5385	0.4415	3.53	4.46
1.562	59.26	150.4	0.6156	0.4118	3.52	4.50
1.923	55.08	150.0	0.7059	0.3828	3.56	4.57
2.240	52.00	149.8	0.7776	0.3614	3.57	4.58
4.564	41.82	148.3	1.287	0.2906	4.19	5.43
5.274	37.51	148.2	1.335	0.2607	3.74	4.84

Mean values $K_0 = 3.48 \cdot 10^{-5}$; $K = 4.56 \cdot 10^{-5}$

tions. The equivalent conductivity was remarkably low and changed only slowly with concentration. The following values were obtained:

$C \cdot 10^3$	3.479	4.567	10.05	14.89	20.58
Λ_c	7.01	6.86	6.46	5.99	5.82

When a limiting conductivity is extrapolated by the formula of KRAUS and BRAY, the value 7.41 is obtained which in no way agrees with the value 157.1, calculated from the equivalent conductivities of the lithium and salicylate ions.

Table 3. Dissociation constant of potassium salicylate.

$\Lambda_0 = 155.6$; $\Lambda_0^* = 147.5$.

(From the data of DIPPY, JENKINS, and PAGE.)

$C \cdot 10^4$	Λ_c	Λ_x	$C_i \cdot 10^4$	α^*	$K_0 \cdot 10^4$	$K \cdot 10^4$
0.7326	125.3	151.1	0.6076	0.8495	2.60	3.76
1.008	120.2	150.4	0.8055	0.8149	2.76	3.62
1.075	119.3	150.2	0.8536	0.8089	2.83	3.68
1.479	111.9	149.5	1.107	0.7587	2.77	3.53
1.693	108.9	149.2	1.234	0.7377	2.77	3.51
2.834	98.90	147.6	1.899	0.6706	3.07	3.87
3.306	92.51	147.2	2.077	0.6272	2.75	3.49
7.360	73.72	144.4	3.759	0.4998	2.85	3.68
11.07	64.03	142.7	4.968	0.4341	2.80	3.69
Mean values $K_0 = 2.80 \cdot 10^{-4}$; $K = 3.65 \cdot 10^{-4}$						

Table 4. Dissociation constant of rubidium salicylate.

$\Lambda_0^* = 150.2$.

$C \cdot 10^3$	Λ_c	α^*	$K \cdot 10^4$
0.2302	107.8	0.7175	4.20
0.4329	90.23	0.6007	3.91
0.6339	80.85	0.5383	3.98
0.6501	80.24	0.5342	3.98
1.005	69.86	0.4651	4.04
1.076	66.55	0.4431	3.79
1.296	62.82	0.4182	3.90
1.608	57.71	0.3842	3.85
2.112	52.97	0.3527	4.06
2.161	51.36	0.3419	3.84
3.234	43.70	0.2909	3.86
4.884	37.54	0.2499	4.07
10.08	27.29	0.1817	4.07
Mean value $K = 3.99 \cdot 10^{-4}$			

This very likely indicates the presence of higher associated complexes. It is thus obvious, that a calculation of the dissociation constant of lithium salicylate in acetone cannot be performed without extending the measurements to extremely low concentrations. The dissociation constants of the salicylates can be summarized thus:

Cation	Li^+	Na^+	K^+	Rb^+	Cs^+	$(CH_3)_4N^+$
$K \cdot 10^4$	< Na	0.456	3.65	3.99	4.56	21.03
$K_0 \cdot 10^4$		0.348	2.80			14.78

Table 5. Dissociation constant of cesium salicylate.

$\Lambda_0^* = 154.2$.

$C \cdot 10^3$	Λ_c	α^*	$K \cdot 10^4$
0.4614	96.03	0.6228	4.75
1.008	73.91	0.4793	4.45
1.247	68.78	0.4461	4.48
1.912	60.32	0.3912	4.81
2.023	57.49	0.3729	4.49
2.519	53.04	0.3440	4.54
3.039	49.03	0.3180	4.51
3.760	45.07	0.2923	4.54
3.955	44.12	0.2861	4.54
4.513	42.08	0.2729	4.62
		Mean value $K = 4.56 \cdot 10^{-4}$	

As seen from this the dissociation constants increase with decreasing chelation tendency of the cation. For tetramethylammonium salicylate it may be supposed that only association into ion pairs takes place.

Summary

When sodium phenoxide is heated together with carbon dioxide under pressure, disodium salicylate and phenol are obtained. Potassium phenoxide under similar conditions gives dipotassium *p*-hydroxybenzoate.

All primary alkali salicylates are decomposed into secondary salt, phenol, and carbon dioxide when heated to higher temperature. Lithium and sodium salicylate thus give secondary salicylates, but potassium, rubidium, and cesium salicylate give secondary *p*-hydroxybenzoates. On the other hand, sodium *p*-hydroxybenzoate, on heating, is partially rearranged into salicylate.

Disodium salicylate can be heated to near 300° without any decomposition, but dipotassium salicylate is completely decomposed into dipotassium *p*-hydroxybenzoate, phenol, and potassium carbonate on heating to 220°.

It is suggested that the different behaviour of the alkali salicylates towards heating can be related to the chelating tendency of the cation. This idea is supported by the observations made on the electrical conductivity of the alkali salicylates in acetone. It was found that the ionization of potassium, rubidium, and cesium salicylate was much greater than the ionization of lithium and sodium salicylate. There is very likely a close connection between the ionization and the chelation. Under this assumption we see that lithium and sodium salicylate, which cannot be rearranged to *p*-hydroxybenzoate, are much more chelated than potassium, rubidium, and cesium salicylate, which are readily converted into *p*-hydroxybenzoate on heating.

Laboratory of Organic Chemistry, University of Uppsala.

REFERENCES

1. Kolbe, J. pr. Chem. [2] *10,* 95 (1874).
2. Silin and Moshchinskaya, J. Gen. Chem. *8,* 810 (1938), C.A. *33,* 1306 (1939).
3. Schmitt and Burkard, Ber. *20,* 2699 (1887).
4. Ref. 1, p. 100.
5. Ost, J. pr. Chem. [2], *11,* 385 (1875).
6. *Org. Synth.* Coll. Vol. *2,* New York 1947, p. 341.
7. Johnson, J. Am. Chem. Soc. *55,* 3029 (1933).
8. Hückel, Theoretische Grundlagen der Organischen Chemie I. Leipzig 1949, p. 290.
9. Fuson, Advanced Organic Chemistry. New York 1950, p. 350.
10. Kupferberg, J. pr. Chem. [2], *13,* 1003 (1876).
11. Martell and Calvin, Chemistry of the Metal Chelate Compounds. New York 1952, p. 394.
12. von den Velden, J. pr. Chem. [2], *15,* 151 (1877).
13. Dippy, Jenkins, and Page, J. Chem. Soc. *1939,* 1386.
14. Fuoss, J. Am. Chem. Soc. *57,* 488 (1935).
15. Felsing and Durban, J. Am. Chem. Soc. *48,* 2890 (1926).
16. Davies, The Conductivity of Solutions. London 1933, p. 270.
17. Kraus and Bray, J. Am. Chem. Soc. *35,* 1315 (1913).
18. Ives, J. Chem. Soc. *1933,* 731.
19. Fuoss and Kraus, J. Am. Chem. Soc. *55,* 476 (1933).
20. Shedlovsky, J. Franklin Inst. *225,* 739 (1938).
21. Walden, Ulich, and Busch, Z. phys. Chem. *123,* 429 (1926).
22. Lannung, Z. phys. Chem. *A 161,* 263 (1932).

Tryckt den 22 maj 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 27

Read 10 March 1954

Studies on synthetic growth substances

VI. Optically active *p*-nitrohydratropic acid and *p*-aminohydratropic acid

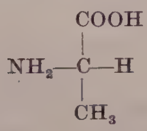
By Arne Fredga

The growth regulating activity of unsubstituted phenylacetic acid and hydratropic (α-phenylpropionic) acid is rather low (1). Comparatively little is known about the influence of substituents in the aromatic ring, but there is reason to believe that substitution in appropriate positions, by halogens for example, may increase the activity. In the series of the aryloxy-acetic acids, such effects have been extensively studied.

Recently, B. Åberg has found that the dextrorotatory hydratropic acid is about 30 times more active in the S-test than the levorotatory form (2). It seems of interest to study the influence of substituents on this steric effect also, and to determine the configuration of the enanthiomorphs of higher activity. The present paper deals with the optical resolution of racemic *p*-nitrohydratropic acid, which is easily effected with α-phenyl-ethylamine and cinchonidine. On reduction, optically active *p*-aminohydratropic acids having the same direction of rotation as the starting material were obtained. Diazotisation and treatment with copper and hypophosphorous acid yielded unsubstituted hydratropic acid. The product was sterically pure and had the same direction of rotation as the amino acid. As the asymmetric carbon atom is not affected by these reactions, the products must have the same configuration as the original hydratropic acid.

Through the diazonium compounds the way is open to various optically active *p*-substituted hydratropic acids, whose configuration relative to hydratropic acid can be definitely established.

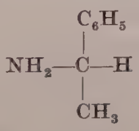
The question of the configuration of hydratropic acid relative to glyceraldehyde has attracted considerable interest. On oxidative degradation of the benzene ring in (−)-α-phenyl-ethylamine, Leithe (3) obtained L-(+)-alanine (I).



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{NH_2{-}C{-}H} \\ | \\ \mathrm{CH_3} \end{array}$$

(+)

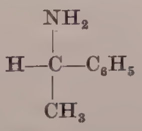
(I)



$$\begin{array}{c} \mathrm{C_6H_5} \\ | \\ \mathrm{NH_2{-}C{-}H} \\ | \\ \mathrm{CH_3} \end{array}$$

(−)

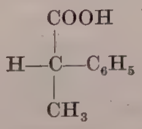
(II)



$$\begin{array}{c} \mathrm{NH_2} \\ | \\ \mathrm{H{-}C{-}C_6H_5} \\ | \\ \mathrm{CH_3} \end{array}$$

(−)

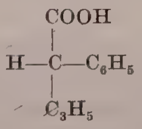
(II a)



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{H{-}C{-}C_6H_5} \\ | \\ \mathrm{CH_3} \end{array}$$

(+)

(III)



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{H{-}C{-}C_6H_5} \\ | \\ \mathrm{C_3H_5} \end{array}$$

(+)

(IV)

The levorotatory amine is thus represented by stereoformula II, which, according to the Fischer convention (4), can also be written as II a. On the other hand, (+)-hydratropic acid can be converted into (−)-α-phenyl-ethylamine by several well-known rearrangement reactions (Curtius, Hofmann, Schmidt, Lossen) (5, 6, 7). According to generally accepted theories, these reactions proceed with retention of the configuration. The (+)-hydratropic acid is thus most probably represented by the stereoformula III, which, if the phenyl group is considered as a substituent to the aliphatic chain, must be regarded as belonging to the D-series.

Additional evidence for this formula was produced by MISLOW and HEFFLER (8), who established the steric connection between hydratropic acid and α-chloro-phenylacetic acid by studying the phase diagrams of the optically active amides. The chloro-phenylacetic acid was obtained from active mandelic acid by treatment with thionyl chloride. This reaction is generally assumed to proceed with retention of the configuration.

There is thus very strong evidence for the D-configuration of the (+)-hydratropic acid. One could, however, object that the evidence is founded on reactions involving substitutions at the asymmetric center and thus postulate the correctness of current theories on the steric course of these reactions.

Recently, the present author has shown (9) that (+)-allyl-phenylacetic acid must have the D-configuration (formula IV). This acid has much higher auxin activity than its enanthiomorph and, as mentioned above, the same holds good for the (+)-hydratropic acid. Regarding the structural similarity of these compounds, it seems justified to assume that the enanthiomorphs with higher auxin activity have the same configuration. We have here a quite independent proof for the D-configuration of the (+)-hydratropic acid.

Table 1

	Molecular rotation (D-line)	
	Hydratropic acid	Allyl-phenyl-acetic acid
Acetone	143.3°	180.5°
Benzene	139.8°	182.1°
Acetic acid	131.1°	161.2°
Ethanol	118.8°	159.1°
Chloroform	113.1°	148.9°
Water (ion)	8.7°	40.2°

Further evidence is obtained by comparing the optical activity data for the two acids in different solvents (Table 1). In both cases the highest values are found for the free acid in benzene or acetone and the lowest for the ion in water.

Optically active hydratropic acid has been prepared and studied by RAPER (10), LEVENE *et al.* (11, 12), OTT and KRÄMER (13), BERNSTEIN and WHITMORE (5), and ARCUS and KENYON (6). It is, however, difficult to compare the optical activity data of the different authors, which in most cases refer to different solvents, concentrations or temperatures (in some cases the temperature is not reported). The present author, in collaboration with Mr. G. ZDANSKY, has therefore tried to prepare the enanthiomorphs in a state of unquestionable purity. The (+)-acid is obtained with strychnine and the (−)-acid with quinine (see experimental part). As the preparations show the same maximum activity and the same melting point, there can hardly be any doubt that they represent the sterically pure acids.

The values of RAPER, $[\alpha]_D^{20} = 81.1°$ (ethanol) and 76.2° (chloroform), are in good agreement with those found by the present author (81.0° and 76.7° respectively). LEVENE and MARKER report $[\alpha]_D^{25}$ 76.0° (benzene), which indicates that their preparation contained only 81–82 % of active acid. The acid of BERNSTEIN and WHITMORE, showing $[\alpha]_D^{25.5} = -86.1°$ (homogeneous), contained about 96 % of active compound. The preparations of maximum activity described by OTT and KRÄMER and by ARCUS and KENYON were sterically pure or very nearly so, the contamination of the other enanthiomorph certainly not exceeding 1–2 %.

Optically active *p*-nitrohydratropic acid has been described earlier by MÜLLER and ILLGEN (14). As seen from the activity and melting point data their preparations contained only about 20 % active acid. They probably represented eutectic mixtures of active and racemic acid.

Addendum to the proof. In the February issue of the Chemische Berichte, which was not accessible to the author when the present paper was sent to the print, NERDEL and FISCHER have described the resolution of *p*-nitrohydratropic acid using (+)-bornylamine. To judge from their data, their best preparations must still have contained about 10 % of racemic acid. They have also, using acid of lower activity, converted the nitro acid into amino acid, unsubstituted hydratropic acid and some derivatives.

Experimental

***p*-Nitrohydratropic acid.** Methyl-benzylcyanid (hydratroponitrile) prepared according to WIDEQVIST (15) was nitrated following the method given by VOGEL (16) for benzylcyanide. The crude product was carefully filtered by suction, washed several times with water and pressed to remove oil by-products. After drying, it was recrystallised twice from petroleum (b.p. 60–80°).

The nitrile was hydrolysed by boiling for 40 minutes with a mixture of equal volumes of concentrated sulphuric acid and water, about 4 ml of each for 1 g of nitrile. The mixture was poured on to an excess of crushed ice and left to stand in a refrigerator over-night. The acid was then filtered off, dried and recrystallised twice from carbon tetrachloride + petroleum. The melting point was 87.5–88.5° in good agreement with the reports of previous authors (17, 18). It did not change on further recrystallisation.

0.2785 g acid: 13.29 ml 0.1074–*N* NaOH.

$C_9H_9O_4N$ Equiv. wt. calc. 195.2 found 195.1

Resolution of the acid into the optical antipodes. Good resolution was obtained with α-phenyl-ethylamine, giving acid of the same direction of rotation as the base. The (+)-acid could also be isolated through the cinchonidine salt. Strychnine and (+)-benzedrine gave dextrorotatory acid but poor resolution, quinine and brucine levorotatory acid of low activity. The acid isolated from the cinchonine salt was quite inactive.

In order to economize the rather expensive α-phenyl-ethylamine, the following procedure was worked out. 58.5 g (0.3 mole) acid, 24.2 g (0.2 mole) (−)-α-phenylethylamine and 5.6 g (0.1 mole) potassium hydroxide were dissolved in a hot mixture of 100 ml ethanol and 50 ml water. 450 ml of hot water were carefully added. The salt was filtered off after 24 hours and recrystallised several times from dilute ethanol; the progress of the resolution was tested on samples from each crystallisation (about 0.3 g), from which the acid was liberated and examined in absolute ethanol.

Crystallisation	1	2	3	4	5	6
ml water.	500	270	200	150	130	115
ml ethanol	100	70	70	60	50	40
g salt obtained . . .	46.8	36.8	30.7	26.2	23.3	21.9
$[\alpha]_D^{25}$ of the acid. . .	$-31.4°$	$-44.9°$	$-49.6°$	$-53.2°$	$-54.6°$	$-54.6°$

The mother liquor from the first crystallisation on standing deposited a second crop of salt which was filtered off and added to a second run. The mother liquor was then evaporated to dryness at room temperature and the acid was liberated. 22.0 g (0.113 mole) having $[\alpha]_D^{25} = +28.3°$ (ethanol) were obtained. The acid was dissolved with 33.2 g (0.113 mole) of cinchonidine in hot dilute ethanol and the resulting salt recrystallised until the acid had reached maximum activity.

Crystallisation	1	2	3	4
ml water	700	400	350	325
ml ethanol	100	100	75	65
g salt obtained	40.9	34.4	30.0	26.8
$[\alpha]_D^{25}$ of the acid	$+47.9°$	$+53.6°$	$+55.6°$	$+54.8°$

(+)-*p*-Nitrohydratropic acid. The cinchonidine salt (26.8 g) was decomposed with dilute sulphuric acid and the organic acid extracted with ether. After evaporation of the ether, the crude acid (10.2 g) was dried and recrystallised three times from carbon tetrachloride. 9.0 g of faintly yellow needles melting at 100–100.5° were obtained.

0.2891 g acid, 13.79 ml 0.1074–*N* NaOH.

$C_9H_9O_4N$ Equiv. wt. calc. 195.2 found 195.3

0.3914 g dissolved in absolute *ethanol* to 10.00 ml: $2\alpha_D^{25} = +4.32°$. $[\alpha]_D^{25} = +55.2°$; $[M]_D^{25} = +107.7°$.

(−)-Nitrohydratropic acid. The α-phenyl-ethylamine salt (21.9 g) was decomposed with dilute sulphuric acid and the organic acid was extracted with ether as described above. The crude acid (13.1 g) was recrystallised from carbon tetrachloride, from ether + petroleum and once more from carbon tetrachloride. 10.3 g of pure acid, melting at 100–100.5°, were obtained.

0.2981 g acid: 14.21 ml 0.1074–*N* NaOH.

$C_9H_9O_4N$ Equiv. wt. calc. 195.2 found 195.3

0.4015 g dissolved in absolute *ethanol* to 10.00 ml: $2\alpha_D^{25} = -4.44°$. $[\alpha]_D^{25} = -55.3°$; $[M]_D^{25} = -107.9°$ — 0.3559 g dissolved in *methanol* to 9.99 ml: $2\alpha_D^{25} = -4.005°$. $[\alpha]_D^{25} = -56.3°$; $[M]_D^{25} = -109.8°$.

***racem-p*-Aminohydratropic acid.** TRINIUS has reduced the *p*-nitrohydratropic acid using tin and hydrochloric acid (17). Reduction with hydrogen sulphide in aqueous ammonia seems, however, more convenient.

19.5 g (0.1 mole) *racem-p*-nitrohydratropic acid were dissolved in a mixture of 80 ml concentrated aqueous ammonia and 80 ml water. The flask was immersed in cold water and the solution saturated with hydrogen sulphide. After standing over night, the mixture was boiled to remove excess of hydrogen sulphide, the precipitated sulphur was filtered off and washed with a little dilute ammonia. When the filtrate was acidified with 12 ml glacial acetic acid, the amino acid separated as glistening plates. The yield of crude acid was 12.3 g. From the mother liquor, a further 2.6 g could be obtained, which was recrystallised from water and added to the main portion. The acid was finally recrystallised twice from water (once with charcoal), yielding 10.6 g. The acid crystallises at higher temperatures in pale yellow, glistening plates or prisms, similar to the crystals of the active compound. At lower temperatures it often crystallises in fine needles. On titration in ethanol solution with aqueous sodium hydroxide and phenolphthalein as indicator, it gives quite a sharp end-point and correct equivalent weight.

0.2364 g acid: 13.30 ml 0.1075–*N* NaOH.
$C_9H_{11}O_2N$ Equiv. wt. calc. 165.2 found 165.3

The acid is obviously dimorphic or polymorphic. TRINIUS gives the m.p. 128° (17). The present author found 136°, but on melting the acid is in most cases transformed to another modification melting at 145–146°. The optically active acid, which has a much higher melting point, displayed no similar anomalies.

(+)-*p*-Aminohydratropic acid. 6.9 g *p*-Nitrohydratropic acid were dissolved in 25 ml concentrated aqueous ammonia + 25 ml water, reduced as described above for the racemic acid and precipitated by adding 4 ml glacial acetic acid. The yield of crude product was 4.5 g; after two recrystallisations from water, there remained 3.4 g. A minor quantity could be isolated from the mother liquors. The acid forms pale yellow, apparently rectangular plates with m.p. 173–174° (capillary introduced at 170°). On slow heating, a slight decomposition can be observed at about 170°.

0.2362 g acid: 13.28 ml 0.1075–*N* NaOH.
$C_9H_{11}O_2N$ Equiv. wt. calc. 165.2 found 165.5
0.2318 g dissolved in absolute *ethanol* to 9.99 ml: $2\alpha_D^{25} = +3.445°$. $[\alpha]_D^{25} = +74.2°$; $[M]_D^{25} = +122.6°$.

(−)-*p*-Aminohydratropic acid. 6.9 g *p*-Nitrohydratropic acid were reduced as described above for the enanthiomorph. The yield of crude product was 4.6 g; after two recrystallisations there remained 3.6 g. In melting point and general appearance it was quite similar to the (+)-acid.

0.2125 g acid: 11.96 ml 0.1075–*N* NaOH.
$C_9H_{11}O_2N$ Equiv. wt. calc. 165.2 found 165.3
0.2276 g dissolved in absolute *ethanol* to 9.98 ml: $2\alpha_D^{25} = -3.38°$. $[\alpha]_D^{25} = -74.1°$; $[M]_D^{25} = -122.4°$.

Resolution of the hydratropic acid. The method followed was essentially that of RAPER (10). The experiment started with 40 g racemic acid and 90 g strychnine in 300 ml ethanol + 100 ml water. The salt obtained (40 g) was recrystallised from 75 % ethanol and the course of the resolution was tested on samples of each fraction, from which the acid was liberated and examined in ethanol solution. After five recrystallisations, maximum activity was obtained. For the sake of control, the salt was recrystallised five times more and the acid was examined for every fraction. The activity remained quite constant. The yield of salt was 11.0 g and 3.2 g after five and ten recrystallisations respectively. The acid was now liberated from the whole portion and recrystallised from a small volume of petrol ether (b.p. 30–50°). The crystallisation must take place at about 0°. The acid was obtained as glassy prisms, melting at 30.2–30.9° (precision thermometer, divided in 0.1° C, efficient stirring of the heating bath, rise of temperature 1° in 4 minutes).

0.1829 g acid: 11.32 ml 0.1075–*N* NaOH.
$C_9H_{10}O_2$ Equiv. wt. calc. 150.2 found 150.3

0.1674 g dissolved in absolute *ethanol* to 10.00 ml: $2\,\alpha_D^{25} = +2.645°$. $[\alpha]_D^{25} = +79.0°$; $[M]_D^{25} = +118.6°$. The rotation was also determined at 20.00°: $2\,\alpha_D^{20} = +2.725°$. After correction for the change in volume this gives: $[\alpha]_D^{20} = +81.0°$; $[M]_D^{20} = +121.6°$.

Systematic fractionation of the strychnine salt from the mother liquors yielded in all 14.3 g salt containing acid of the same maximum activity.

16.5 g acid recovered from the first mother liquors of the strychnine salt and containing considerable excess of (–)-form was dissolved with 35.7 g quinine in 800 ml acetone; 25 g salt were obtained. It was recrystallised six times; the activity of the acid remained constant from the fourth recrystallisation. The yield was 9.3 g and 6.3 g after four and six crystallisations respectively. The acid was liberated and recrystallised as described above. The melting point was 30.3–31.0°.

0.2270 g acid: 14.08 ml 0.1075–*N* NaOH.
$C_9H_{10}O_2$ Equiv. wt. calc. 150.2 found 150.0

0.1567 g dissolved in absolute *ethanol* to 10.00 ml: $2\,\alpha_D^{25} = -2.48°$. $[\alpha]_D^{25} = -79.1°$; $[M]_D^{25} = -118.8°$. — 0.1544 g dissolved in *benzene* to 10.00 ml: $2\,\alpha_D^{25} = -2.875°$. $[\alpha]_D^{25} = -93.1°$; $[M]_D^{25} = -139.8°$. — 0.1482 g dissolved in *acetone* to 9.98 ml: $2\,\alpha_D^{25} = -2.835°$. $[\alpha]_D^{25} = -95.5°$; $[M]_D^{25} = -143.3°$. — 0.1801 g dissolved in glacial *acetic acid* to 9.99 ml: $2\,\alpha_D^{25} = -3.145°$. $[\alpha]_D^{25} = -87.3°$; $[M]_D^{25} = -131.1°$. — 0.1587 g dissolved in *chloroform* to 10.00 ml: $2\,\alpha_D^{25} = -2.39°$; $[\alpha]_D^{25} = -75.3°$; $[M]_D^{25} = -113.1$. The rotation was also determined at 20.00°: $2\,\alpha_D^{20} = -2.45°$. After correction for the change in volume this gives: $[\alpha]_D^{20} = -76.7°$; $[M]_D^{20} = -115.3°$.

The rotatory power of the ion in aqueous solution given in Table 1 is quoted from ARCUS and KENYON (6). The data for the α-allyl-phenylacetic acid are taken from PICKARD and YATES (10) and from FREDGA and WESTMAN (9).

(–)-Hydratropic acid from (–)-*p*-aminohydratropic acid. 1.6 g (0.01 mole) (–)-*p*-aminohydratropic acid was suspended in 25 ml 4-*N* sulphuric acid and diazotised with 0.7 g sodium nitrite dissolved in 5 ml water. The diazonium solution was added to a cooled suspension of "copper hydride" prepared from 10 g crystallised copper sulphate, and 20 g hypophosphorous acid in 200 ml water according to VORLÄNDER and MEYER (20). After standing over night, 5 g hypophosphorous acid were added and the mixture was heated to 50–60° for 15 minutes, cooled, filtered and extracted with benzene. After drying, the benzene was distilled off, leaving a colourless syrup which crystallised in the refrigerator. It was recrystallised twice from a very small volume of petrol ether (b.p. 30–50°). The yield was 0.44 g and the melting point 30.0–30.6° (no depression in mixture with an authentic sample of (–)-hydratropic acid).

0.1614 g acid: 9.95 ml 0.1075-*N* NaOH.
$C_9H_{10}O_2$ Equiv. wt. calc. 150.2 found 150.9

0.2053 g dissolved in absolute *ethanol* to 10.00 ml: $2\alpha_D^{25} = -3.25°$. $[\alpha]_D^{25} = -79.2°$; $[M]_D^{25} = -118.9°$.

Summary

p-Nitrohydratropic acid has been resolved into the optical antipodes showing $[\alpha]_D^{25} = +55.2°$ and $-55.3°$ (in ethanol).

Reduction with hydrogen sulphide in aqueous ammonia gave *p*-aminohydratropic acid having the same direction of rotation as the original *p*-nitro acid. The rotatory power found was $[\alpha]_D^{25} = +74.2°$ and $-74.1°$ (in ethanol).

On diazotisation and elimination of the diazonium group, (−)-*p*-aminohydratropic acid gave sterically pure (−)-hydratropic acid. The configuration and maximum rotation of the hydratropic acid are discussed.

ACKNOWLEDGEMENTS

The author is indebted to Mr. G. Zdansky for valuable assistance with the resolution of the hydratropic acid and to Mr. R. Öhberg for preparing a large quantity of hydratroponitrile. The expenses involved in this investigation have been defrayed by a grant from the *Nobel Fund of the Royal Swedish Academy of Science* (*Kemiska prisgruppens särskilda fond*).

Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala, March 1954.

REFERENCES

1. Veldstra, H., Ann. Rev. of Plant Physiol. *4*, 151 (1953).
2. Åberg, B., Private communication.
3. Leithe, W., Ber. *64*, 2827 (1931).
4. Fischer, E., Ber. *24*, 2683 (1891).
5. Bernstein, H. I. and Whitmore, F. C., J. Am. Chem. Soc. *61*, 1324 (1939).
6. Arcus, C. L. and Kenyon, J., J. Chem. Soc. *1939*, 916.
7. Campbell, A. and Kenyon, J., J. Chem. Soc. *1946*, 25.
8. Mislow, K. and Heffler, M., J. Am. Chem. Soc. *74*, 3668 (1952).
9. Fredga, A. and Westman, L., Arkiv Kemi, *7*, 193 (1954).
10. Raper, H. S., J. Chem. Soc. *1923*, 2557.
11. Levene, P. A. and Marker, R. E., J. Biol. Chem. *100*, 692 (1933).
12. Rothen, A. and Levene, P. A., J. Chem. Phys. *7*, 975 (1939).
13. Ott, E. and Krämer, K., Ber. *68*, 1657 (1935).
14. Müller, E. and Illgen, R., Ann. *521*, 77 (1936).
15. Wideqvist, S., Svensk Kem. Tid. *55*, 125 (1943).
16. Vogel, A. I., Practical Organic Chemistry, London 1951, p. 723.
17. Trinius, P., Ann. *227*, 262 (1885).
18. Nerdel, F. and Kresze, G., Chem. Ber. *85*, 168 (1952).
19. Pickard, R. H. and Yates, J., J. Chem. Soc. *95*, 1011 (1909).
20. Vorländer, D. and Meyer, F., Ann. *320*, 143 (1902).

Tryckt den 22 maj 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 28

Communicated 10 February 1954 by ARNE FREDGA

On mercaptothiadiazines and *N*-aminothiazolethiones

By JAN SANDSTRÖM

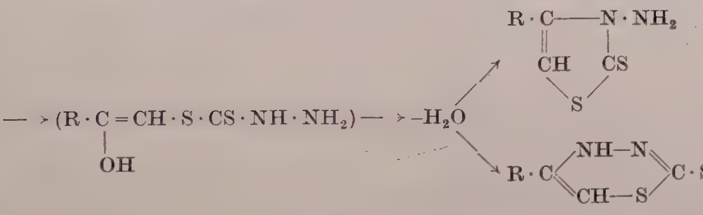
1. Introduction and survey

As a continuation of an investigation of ethyldithiocarbhydrazide and its derivatives (1), the reactions between ammonium dithiocarbazinate and monochloroacetone and between ammonium dithiocarbazinate and phenacyl bromide have been examined.

In the first reaction, neither a crystalline acetonyldithiocarbhydrazide (I a) nor a crystalline benzaldehyde hydrazone thereof could be obtained. Instead, the reaction mixture always contained two anhydrides of (I a), formed according to the reaction scheme (I) or some variant thereof, namely 3-amino-4-methyl-2(3)-thiazolethione (II a), m.p. 94°–95°, which is insoluble in aqueous alkali, and 2-mercapto-5-methyl-1,3,4,4H-thiadiazine (III a), m.p. 126°–127°, which is easily soluble in aqueous alkali. The latter compound has recently been described by MATHES (2), who obtained it by hydrolysis and decarboxylation of ethyl 2-mercapto-5-methyl-1,3,4,4H-thiadiazine-6-carboxylate (IV) and reports m.p. 124.5°–125.5°.

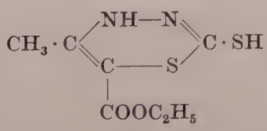
I $RCOCH_2Hal + H_4NS \cdot CS \cdot NH \cdot NH_2 \longrightarrow RCOCH_2S \cdot CS \cdot NH \cdot NH_2 \longrightarrow$

(I a, $R = CH_3$)
I b, $R = C_6H_5$

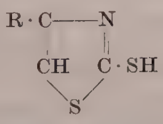


II a, $R = CH_3$
II b, $R = C_6H_5$

III a, $R = CH_3$
III b, $R = C_6H_5$



(IV)



V a, $R = CH_3$
V b, $R = C_6H_5$

The structure of (II a) was demonstrated in the following way. It must contain an amino group, since it gave Schiff bases with aromatic aldehydes. The compound with benzaldehyde could not be obtained in a pure state, but the compound with veratraldehyde, m.p. 134°–135°, crystallized well and could be easily purified. That this amino group is attached to the nitrogen in a 4-methyl-2(3)-thiazolethione moiety was shown by the reaction with nitrous acid. In this reaction, amino groups attached to nitrogen in heterocyclic rings are exchanged for hydrogen (3, 4). In this special case, however, the reaction product was not 2-mercapto-4-methylthiazole (V a) but its disulphide, formed by oxidation of the thiol by excess of nitrous acid.

The isomeric anhydride (III a) was made to react in alkaline solution with monochloroacetate, but on acidification only sticky products were obtained. When this anhydride was left in contact with dilute hydrochloric acid for two days, it was quantitatively transformed into the other anhydride (II a).

In the reaction between ammonium dithiocarbazinate and phenacyl bromide, the primary product was phenacyldithiocarbhydrazide (I b), m.p. 126°–127°, which with benzaldehyde gave a well crystallized hydrazone, m.p. 172°–173°. When heated above its melting point or refluxed with ethanolic hydrochloric acid, (I b) was transformed into a mixture of two anhydrides, 3-amino-4-phenyl-2(3)-thiazolethione (II b), m.p. 146°–147°, and 2-mercapto-5-phenyl-1,3,4,4H-thiadiazine (III b), m.p. 136°–137°. Like the corresponding methyl derivatives, (II b) was insoluble and (III b) was easily soluble in aqueous alkali. The anhydride (III b) also has recently been described by MATHES (2), who obtained it by mixing cooled solutions of ammonium dithiocarbazinate and phenacyl bromide, heating the resulting mixture with hydrochloric acid and recrystallizing the product from ether. He reports m.p. 133°–134°.

When (I b) was refluxed with ethanolic hydrochloric acid, the anhydride (II b) formed one-third of the resulting mixture, but when (I b) was heated above its melting point, (II b) formed three-fourths of the mixture. With benzaldehyde (II b) gave a well crystallized Schiff base, m.p. 115°–116°, and in reaction with nitrous acid it gave the disulphide of the 2-mercapto-4-phenylthiazole (V b).

When the anhydride (III b) was dissolved in aqueous alkali and made to react with monochloroacetate, the salt of an acid was obtained. However, this acid was too unstable to be purified and it was therefore not further investigated. When iodine was added to an alkaline solution of (III b), a disulphide was obtained, but this too was unstable and could not be purified.

When the anhydride (III b) was refluxed with ethanolic hydrochloric acid, it was slowly rearranged to (II b), but the reverse reaction could not be effected.

Recently, BEYER and coworkers (5) have examined the reaction between monochloroacetone and thiosemicarbazide. They were able to prepare the S-acetonyl-*iso*thiosemicarbazide and the three possible anhydrides thereof.

2. Experimental

A. Products of ammonium dithiocarbazinate and monochloroacetone

a) *Anhydrides of acetonyldithiocarbhydrazide*

A solution of 6.3 g of ammonium dithiocarbazinate[1] in 10 ml of water was cooled with ice and salt, and a solution of 4.6 g of monochloroacetone in 30 ml of ethanol was added by drops with stirring. The mixture became turbid but was soon clear and separated a yellow oil, which gradually became a dark brown resin, weighing 0.8 g. The supernatant clear solution was decanted off, and one-fifth thereof, 9 ml, was mixed with 1.1 g of benzaldehyde. A clear, yellow mixture was obtained, which soon became turbid and separated a yellow oil, which gradually darkened and could not be made to crystallize.

---

[1] Prepared according to LOSANITCH (6).

The rest of the solution was immediately (one hour after starting the reaction) evaporated in a vigorous stream of air. After five hours the remaining brown crystal mass was ground with water, sucked off and air dried. The residue weighed 4.9 g and melted at about 60°–75°. It was ground with 40 ml of *N*-NaOH and gave an undissolved residue of 3.0 g of brown crystal powder, m.p. 93°–95° (after sintering). When recrystallized from 350 ml of ligroine (b.p. 80°–100°) the latter product gave 2.6 g of long, colourless, rod-shaped prisms, m.p. 94°–95°, of 3-amino-4-methyl-2(3)-thiazolethione (II a).

For $C_4H_6N_2S_2$ (146.23)

calc.:	C 32.9	H 4.14	N 19.16	S 43.9 %,
found:	C 32.9*[1]	H 4.12*	N 19.13	S 43.9* %.

When the alkaline solution was acidified with acetic acid, 1.0 g of brown, rod-shaped prisms separated, m.p. 119°–123°. The product was recrystallized from 200 ml of ligroin (b.p. 80°–100°) and gave 0.6 g of pale yellow, rod-shaped prisms, m.p. 125°–126°. A further recrystallization from 3 ml of ethanol gave 0.4 g of colourless, rod-shaped prisms, m.p. 126°–127°, of 2-mercapto-5-methyl-1,3,4,4H-thiadiazine (III a), isomeric with (II a).

found: C 32.9* H 3.96* %.[1]

On attempting to titrate this thiol against standard alkali with phenolphthalein or against standard iodine with starch, extremely blunt and sliding end points were obtained, when about three fourths of the calculated amounts of the standard solutions had been added.

When 0.5 g of the thiol was dissolved in 4 ml of *N*-NaOH, precipitated with 1 ml of 5 *N*-HCl and immediately sucked off, the precipitate, 0.5 g, had m.p. 126°–127°, but if it was allowed to remain in contact with the acid solution for two days, the resulting product had the same weight but m.p. 94°–95° alone and also on admixture with (II a).

b) *3-Veratrylidenamino-4-methyl-2(3)-thiazolethione*

A solution of 0.8 g of (II a) and 0.8 g of veratraldehyde[2] in 5 ml of ethanol remained clear and colourless during 24 hours, but when 1 ml of concentrated hydrochloric acid was added, the solution turned yellow and soon became turbid and separated an oil, which presently solidified to 1.4 g of a yellow crystalline mass, m.p. 132°–134°. The product was recrystallized from 10 ml of ethanol and gave 1.2 g of yellow, rod-shaped prisms, m.p. 134°–135°. A further recrystallization from ethanol did not change the m.p.

---

[1] The analyses marked with * have been performed by Miss M. WESTERDAHL at the Analytical Department, University of Lund, and the rest by the author.

[2] When (II a) was made to react in the same way with benzaldehyde, an oil was obtained, which could only be made to crystallize after a long time. On recrystallization from ethanol the product separated as a slowly crystallizing oil in spite of seeding. After three recrystallizations it was still rather impure. The m.p. was then 88°–90°.

For $C_{13}H_{14}O_2N_2S_2$ (294.38)
calc.: C 53.0 H 4.79 N 9.52 S 21.8 %,
found: C 53.1* H 4.77* N 9.52 S 21.9* %.

c) *The reaction between 3-amino-4-methyl-2(3)-thiazolethione and nitrous acid*

A cooled solution of 1.4 g of sodium nitrite in 10 ml of water was added dropwise with stirring to a cooled suspension of 1.5 g of aminomethylthiazolethione in 5 ml of 5 *N*-HCl. When the evolution of gas had ceased, the mixture was turbid and soon separated crystals, and until next day 1.0 g of long, brown-yellow plates, m.p. 59–61°, had formed. The product was recrystallized from 20 ml of petroleum ether (b.p. 40°–60°) and gave 0.7 g of colourless plates, m.p. 62°–63°, according to analyses consisting of bis-[4-methylthiazol-2-yl]-disulphide.

For $C_8H_8N_2S_4$ (260.40)
calc.: C 36.9 H 3.10 N 10.76 S 49.2 %,
found: C 37.0* H 3.14* N 10.78 S 49.2* %.

This product has previously been described by BUCHMAN *et al.* (7), who obtained it by oxidizing methylmercaptothiazole (V a) with hydrogen peroxide. They report m.p. 61°–61.5°. An authentic sample of the disulphide was prepared by oxidizing methylmercaptothiazole[1] with iodine and recrystallizing the product from petroleum ether (b.p. 40°–60°). The pure product had m.p. 62°–63° alone and also on admixture with the disulphide described above.

B. Products of ammonium dithiocarbazinate and phenacyl bromide

a) *Phenacyldithiocarbhydrazide*

A solution of 19.9 g of phenacyl bromide in 150 ml of ethanol was added dropwise with cooling and stirring to a solution of 12.5 g of ammonium dithiocarbazinate in 60 ml of water. The mixture became turbid and gradually deposited a pale-yellow crystal mass, the amount of which was further increased on the addition of 200 ml of water. By the following day 19.0 g (calc. 22.6 g) of pale-yellow, irregular prisms had separated, m.p. 116°–120°. This product was used for the reactions described later. On attempts at purification the crude product was found to be very sensitive to heat, whereas the pure product could be dissolved in boiling benzene without decomposition. The purification was accomplished in the following way. The crude hydrazide, 2.0 g, was dissolved in 20 ml of ethanol at 20°, the solution was cooled to − 15° and 20 ml of water was carefully added. The mixture was turbid and 1.3 g of colourless, irregular prisms separated, m.p. 119°–121°, and when this product was precipitated in the same way from a solution in 15 ml of ethanol with 15 ml of water, the resulting product weighed 0.9 g and showed m.p. 122°–123°. From this product 0.8 g was dissolved in 15 ml of benzene at 50°, and on cooling the solution with ice 0.6 g of colourless crystal powder separated, m.p. 124°–125°. This product, finally, was dissolved in 10 ml of boiling benzene, and on cooling the solution 0.5 g of colourless rod-shaped prisms separated, m.p. 126°–127°, according to analyses consisting of phenacyldithiocarbhydrazide (I b).

---

[1] Prepared according to MIOLATI (8).

For $C_9H_{10}ON_2S_2$ (226.31)

calc.: C 47.8 H 4.45 N 12.38 S 28.3 %.
found: C 47.7* H 4.37* N 12.42 S 28.4* %.

The filtrate from the crude product on the following day had separated 0.7 g of long, yellow rods, m.p. 134°–136°, which were recrystallized from 7 ml of ethanol to give 0.5 g of yellow flakes, m.p. 136°–137°, consisting of mercaptophenylthiadiazine (III b) to be described below.

b) *Benzaldehyde phenacyldithiocarbhydrazone*

When 2.2 g of benzaldehyde was added to a solution of 4.6 g of phenacyldithiocarbhydrazide in 30 ml of ethanol, the temperature rose to about 40° and the mixture soon began to separate crystals. On the following day 5.3 g of pale-yellow flakes, m.p. 171°–172°, had formed. The product was very slightly soluble in boiling ethanol and in boiling benzene, but it could easily be recrystallized from a mixture of 50 ml of absolute ethanol and 50 ml of benzene. The resulting product was 3.2 g of very pale-yellow plates, m.p. 172°–173°, according to analyses consisting of benzaldehyde phenacyldithiocarbhydrazone.

For $C_{16}H_{14}ON_2S_2$ (314.41)

calc.: C 61.1 H 4.49 N 8.91 S 20.4 %,
found: C 61.1 H 4:48 N 8.95 S 20.4* %.

On attempting to oxidize this product with iron(III)chloride or to make it react with 2,4-dinitrophenylhydrazine no definable products could be obtained. The hydrazone could be dissolved in *N*-NaOH, but the solution was unstable, and on reaction with monochloroacetate no definable product could be obtained.

c) *The anhydrides of phenacyldithiocarbhydrazide*

When 4.6 g of phenacyldithiocarbhydrazide was refluxed for 15 minutes with 5 ml of concentrated hydrochloric acid and 50 ml of ethanol, a clear yellow solution was obtained. After cooling, the solution was poured into 200 ml of water, which caused the separation of a yellow oil. On scratching, the oil solidified to 4.1 g of a yellow crystalline mass, m.p. 104°–106° (after some sintering). The product was ground with 20 ml of *N*-NaOH and gave an undissolved residue of 1.3 g of pale-yellow powder, m.p. 139°–142° (after sintering). The product was recrystallized twice from 20 ml of ethanol and gave 0.9 g of colourless flakes, m.p. 146°–147°, consisting of 3-amino-4-phenyl-2(3)-thiazolethione (II b).

For $C_9H_8N_2S_2$ (208.29)

calc.: C 51.9 H 3.87 N 13.45 S 30.8 %,
found: C 51.8 H 3.80 N 13.50 S 30.7* %.

The strongly yellow alkaline filtrate from this product on acidification separated 2.7 g of yellow flakes, m.p. 132°–134°. After recrystallization twice from 25 and 20 ml of ethanol the product was 2.0 g of yellow flakes, m.p. 136°–137°, of 2-mercapto-5-phenyl-1,3,4,4H-thiadiazine (III b), isomeric with (II b).

found: C 51.7 H 3.78 N 13.49 S 30.8* %.

On attempting to titrate this thiol against standard alkali with phenolphthalein or against standard iodine with starch, extremely blunt and sliding end points were obtained, when about three-fourths of the calculated amounts of the standard solutions had been added.

The two anhydrides (II b) and (III b) could also be prepared by heating the phenacyldithiocarbhydrazide above its melting point. Solid phenacyldithiocarbhydrazide, 4.6 g, was heated in a metal bath at 135°, when the solid melted and gave off water with foaming. When, after five minutes, the fluid was clear and no more bubbles were formed, the flask contents were cooled and solidified to a yellow crystalline mass. This was ground with 20 ml of *N*-NaOH and gave an undissolved residue of 3.1 g of pale-yellow powder, m.p. 139°–140° (after sintering), which was recrystallized twice from 50 and 40 ml of ethanol to give 2.0 g of colourless flakes, m.p. 146–147°. From the alkaline solution on acidification was obtained 1.1 g of yellow flakes, m.p. 132°–134°, recrystallized twice from 10 and 8 ml of ethanol to give 0.7 g of yellow flakes, m.p. 136°–137°.

When the thiol (III b) was refluxed with ethanolic hydrochloric acid it was slowly rearranged to (II b) A solution of 2.1 g of mercaptophenylthiadiazine and 2 ml of concentrated hydrochloric acid in 30 ml of ethanol was refluxed for seven hours and then the mixture was poured into 150 ml of water. The mixture was turbid and separated a yellow oil, which rapidly solidified. The product was ground with 10 ml of *N*-NaOH and gave an undissolved residue of 0.8 g of pale-brown powder, m.p. 143°–145°, which was recrystallized from 15 ml of ethanol and gave 0.6 g of colourless flakes, m.p. 146°–147° alone and also on admixture with (II b). The alkaline solution on acicification separated 1.2 g of yellow flakes, m.p. 134°–135°, which were recrystallized from 10 ml of ethanol and gave 1.0 g of yellow flakes, m.p. 136°–137° alone and also on admixture with pure starting material.

When the aminophenylthiazolethione (II b) was refluxed with ethanolic hydrochloric acid no rearrangement could be observed but only a slight decomposition.

d) *3-Benzylidenamino-4-phenyl-2(3)-thiazolethione*

The anhydride (II b) could not be made to react with benzaldehyde in ethanolic solution but did so readily when the components were dissolved in ethanolic hydrochloric acid or in glacial acetic acid. Aminothiazolethione, 0.5 g, and benzaldehyde, 0.3 g, were dissolved in 5 ml of hot glacial acetic acid, and on cooling, 0.6 g of yellow rod-shaped prisms separated, m.p. 113°–114°. The product was recrystallized from 5 ml of ethanol and gave 0.4 g of yellow, rod-shaped prisms, m.p. 115°–116°. A further recrystallization from ethanol did not change the melting point.

For $C_{16}H_{12}N_2S_2$ (296.40)

calc.:	C 64.8	H 4.08	N 9.45	S 21.6 %,
found:	C 64.8	H 4.17	N 9.47	S 21.6* %.

This product was also obtained together with mercaptophenylthiadiazine (III b), both in a very impure state, when the benzaldehyde phenacyldithiocarbhydrazone was refluxed with ethanolic hydrochloric acid. When 2.0 g of the hydrazone was refluxed for 15 minutes, 1.1 g of the benzylidene derivative described above and 0.5 g of the mercaptophenylthiadiazine were obtained (crude products).

e) *The reaction between 3-amino-4-phenyl-2(3)-thiazolethione and nitrous acid*

Solid sodium nitrite, 1.0 g, was added in portions with grinding to a suspension of 0.9 g of aminophenylthiazolethione in 10 ml of glacial acetic acid. When the evolution of gas had ceased, the mixture was poured into 100 ml of water, which caused the separation of 0.8 g of colourless crystal powder, m.p. 153°–154° (after sintering). The product was recrystallized twice from a mixture of 5 ml of benzene and 10 ml of absolute ethanol and gave 0.7 g of colourless, thin, rod-shaped prisms, m.p. 158°–159°, according to analyses consisting of bis-[4-phenylthiazol-2-yl]-disulphide.

For $C_{18}H_{12}N_2S_4$ (384.54)

calc.:	C 56.2	H 3.15	N 7.29 %,
found:	C 56.4	H 3.22	N 7.30 %.

This disulphide has earlier been described by LEVI (9), who obtained it by oxidizing phenylmercaptothiazole (V b) with iodine and reports m.p. 154°. The thiol was prepared according to MIOLATI (8), who reports m.p. 168°. When MIOLATI's preparation was repeated, a phenylmercaptothiazole was obtained with m.p. 175°–176° and the equiv. w. 191 against standard alkali as against standard iodine (calc. 193.3), and when this was oxidized with iodine a disulphide was obtained with m.p. 158°–159° (after recrystallization from a mixture of benzene and absolute ethanol) alone and also on admixture with the disulphide described above.

Department of Organic Chemistry, University of Lund, Lund, Sweden.

REFERENCES

1. SANDSTRÖM, J., Arkiv Kemi *4*, 297 (1952).
2. MATHES, R. A., J. Org. Chem. *17*, 877 (1952).
3. CURTIUS, TH., *et al.*, Ber. *40*, 836 (1907).
4. HOGGARTH, E., J. Chem. Soc. *1952*, 4811.
5. BEYER, H., *et al.*, Chem. Ber. *86*, 764 (1953).
6. LOSANITCH, S., J. Chem. Soc. *119*, 763 (1921).
7. BUCHMAN, E., *et al.*, J. Org. Chem. *6*, 764 (1941).
8. MIOLATI, A., Gazz. chim. ital. *23*, 575 (1893); C. *1893* II, 426.
9. LEVI, T. G., Gazz. chim. ital. *61*, 719 (1931); C. A. *26*, 1602 (1932).

Tryckt den 22 maj 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 29

Vorgelegt am 27. Januar 1954

Untersuchungen am Yoshida-Ascites-Sarkom der Ratte

Von Hans von Euler, Beth von Euler und Hans Hasselquist

Mit 2 Figuren im Text

Die Entstehung des *Yoshida-Ascites-Sarkoms*[1] geht zurück auf die Behandlung von Ratten mit einen cancerogenen Azofarbstoff, dem Dimethyl-aminoazobenzol (Buttergelb). Schon insofern schien ein Zusammenhang dieses Sarkoms mit Buttergelb-Hepatomen gegeben, und neuerdings erhielten Makino, Tanaka und Kano[2] aus einem dem Yoshida-Sarkom ähnlichen, durch o-Amino-Azotoluol hervorgerufenen Ascites-bildenden Tumor ein Hepatom.[3] Die neuen Yoshida-Sarkom-ähnlichen Stämme sind MTK-Sarkome benannt worden.

Bei der ersten Hervorrufung des Yoshida-Sarkoms wurden die mit Buttergelb vorbehandelten Ratten 3 Monate mit einer Lösung von Kalium-arsenit am Rücken gepinselt. Nach dieser Zeit entstand ein fester Tumor, der sich in das Peritoneum ausbreitete, und dort einen dicken, milchigen Ascites hervorrief, der sich gut transplantieren liess.

Das von uns bearbeitete Yoshida-Sarkom entstammt dem Institut des Entdeckers. Wir erhielten diesen interessanten in Freiburg weiter gezüchteten Tumor von Herrn Prof. Dr. H. Druckrey, dem wir dafür zu grossem Dank verpflichtet sind. Dieses Yoshida-Sarkom wurde hier mit gutem Erfolg weiter gezüchtet, teils durch Überimpfung des Ascites, teils durch Schnitt-Impfung des festen Tumors, und zwar sowohl in unseren norwegischen, reinen Albino-Stamm als in den gelben BDI-Stamm.

Das Yoshida-Ascites-Sarkom entwickelt sich an unserem reinen norwegischen Albino-Stamm ebenso gut wie an dem gelben Stamm BD I der gelben-Ratten (erhalten von der Zucht Prof. H. Druckreys, Freiburg), sowie an den durch die Paarung dieser beiden Stämme[5] erhaltenen Nachkommen (F_1-Generation).

---

[1] Tomizo Yoshida, Y. Muta u. Z. Sasaki, Proc. Imp. Acad. Tokyo 20, 611 (1944). — Gann, 40, 1 (1949).

[2] S. Makino, T. Tanaka u. K. Kano, Gann 42, 199 (1951).

[3] T. Tanaka u. K. Kano, Journ. Fac. Sci. Hokkaido Univ. VI, 10, 289 (1951).

[4] S. Makino u. T. Yoshida, Journ. Fac. Sci. Hokkaido Univ. VI, 10, 209 (1951).

[5] Wie an anderer Stelle besprochen werden soll, erhielten wir durch die Paarung der gelben Rasse mit unserem Albino-Stamm folgende Pelz-Farben in der Nachkommenschaft:

$$\underbrace{\text{Weiss} \times \text{Gelb}}_{\text{alle Junge dunkelgrau; dunkle Iris}}$$

Diese dunkelgraue F_1-Generation wurde folgendermassen gepaart:

dunkelgrau × ♀ dunkelgrau	♂ dunkelgrau × ♀ weiss	♂ gelb × ♀ dunkelgrau	♀ dunkelgrau × ♂ gelb
von 25 Jungen	von 41 Jungen	von 40 Jungen	von 40 Jungen
13 schwarze	23 schwarze	21 dunkelgrau	19 dunkelgrau
4 gelbe	18 weisse	19 gelb	21 gelb
8 weisse	0 gelbe		

Die in der F_2-Generation mit „schwarz“ bezeichneten Tiere sind teils dunkelgrau (wie die ganze F_1-Generation), teils mehr oder weniger rein schwarz.

In Japan ist das Yoshida-Ascites-Sarkom (YAS) an Ratten durch T. Yoshida selbst und durch seine Mitarbeiter und Kollegen[1, 2], in Deutschland besonders durch H. Lettré[3] sowie durch H. Druckrey[4] in cytologischer Hinsicht eingehend untersucht worden. An Mäusen hat G. Klein[5] eine Reihe verschiedener Ascites-Tumoren, darunter auch das Yoshida-Ascites-Sarkom, in bemerkenswerten Versuchen studiert. Zu erwähnen sind auch noch die neuen Arbeiten von Goldie und Felix[6] sowie bezgl. anderen Ascites-Materials von Seeger[7].

Zur Methodik

Gewinnung des Yoshida-Ascites-Sarkoms: Wir entnehmen den Ratten die Ascites-Flüssigkeit durch Aufsaugen mittels Injektions-Spritzen und überführen in der Regel etwa 0,5 ml sofort intraperitoneal in die Versuchstiere. Das Ascites-Sarkom geht beinahe 100prozentig an und entwickelt sich regelmässig; Retrogradation haben wir bisher nicht beobachtet. Zu Beginn unserer Untersuchung haben wir 1–2 ml Ascites-Flüssigkeit eingespritzt; in diesen Fällen führte die Behandlung in 6–8 Tagen zum Tod. Auch die Schnitt-Impfungen in die Bauchhöhle gingen beinahe 100prozentig an.

Sowohl nach Einspritzung der Ascites-Flüssigkeit wie auch nach der Schnitt-Impfung verbreitete sich ein fester Tumor über einen grossen Teil der Bauchhöhle, und zwar besonders an Mesenterium und Pankreas.

Entwicklungszeit des festen Sarkoms: Etwa am 3. Tag nach Einführung der Ascites-Flüssigkeit wird das feste Sarkom deutlich wahrnehmbar, und nimmt bis zum Tod des Tieres am 8.–10. Tag zu.

Die kurz nach der Injektion entnommene Flüssigkeit enthält die eingespritzten und die neugewachsenen Tumorzellen verschiedenen Alters und dementsprechend verschiedener Vermehrungsfähigkeit.

Die Zunahme der YAS-Flüssigkeit nach der Injektion wird am einfachsten verfolgt durch Wägung der Tiere (Vergleich mit der Gewichtszunahme gleichalteriger Normaltiere), oder durch Entnahme einer kleinen Menge Ascites-Flüssigkeit aus dem lebenden Tier und Zählung der Sarkomzellen nach dem für Erythrozyten-Zählung üblichen Verfahren.

Man kann in dieser Weise bei Injektion einer gewissen Menge Sarkomzellen ihre Vermehrungsfähigkeit (Virulenz; Resistenz) und auch die Wachstumsbeeinflussung durch cancerostatische Stoffe messen. G. Klein, der solche Versuche mit Ehrlich-Ascites-Tumor angestellt hat (Akad. Abhandl.), bespricht S. 19 u. 29 die Vorteile und Nachteile dieses rel. schnell durchführbaren, zu orientierenden Messungen jedenfalls sehr geeigneten Verfahrens.

Wir saugen in einer Injektions-Spritze aus dem Ascites-Träger etwa 0,3 ml Ascites-

---

[1] S. Makino u. H. Nakahara, Z. Krebsforsch. 59, 298 (1953). — K. Takeda et al., Gann 43, 132 (1952).

[2] S. Amano, Monographie, Japan 1948. — Y. Hamazaki, Acta Hematol. Japan. 12, 136 (1949). — K. Takeda, K. Nakamura, S. Mori, H. Kumagai, K. Shimizu u. K. Ikeda, Gann 40, 120 (1949).

[3] H. Lettré, Z. Krebsforsch. 59, 287 (1953); Z. physiol. Chem. 268,59 (1941); 271, 190 (1941); Z. Krebsforsch. 57, 1, 142 (1950).

[4] H. Druckrey, Dtsch. Med. Woch. 77, 1495 (1952). — Arzneim. Forsch. 3, 151 (1953).

[5] G. Klein, Akad. Abhandl. Stockholm 1951. — L. Goldberg, E. Klein u. G. Klein, Exper. Cell Research 1, 543 (1950). — G. Klein, Cancer 3, 1052 (1950). — G. Klein u. E. Klein, Cancer Research 11, 466 (1951). — G. Klein, Exper. Cell Research 2, 518 (1951).

[6] H. Goldie and M. D. Felix, Cancer Research 11, 73 (1951).

[7] P. G. Seeger, Arch. exper. Zellforsch. 22, 356 (1938).

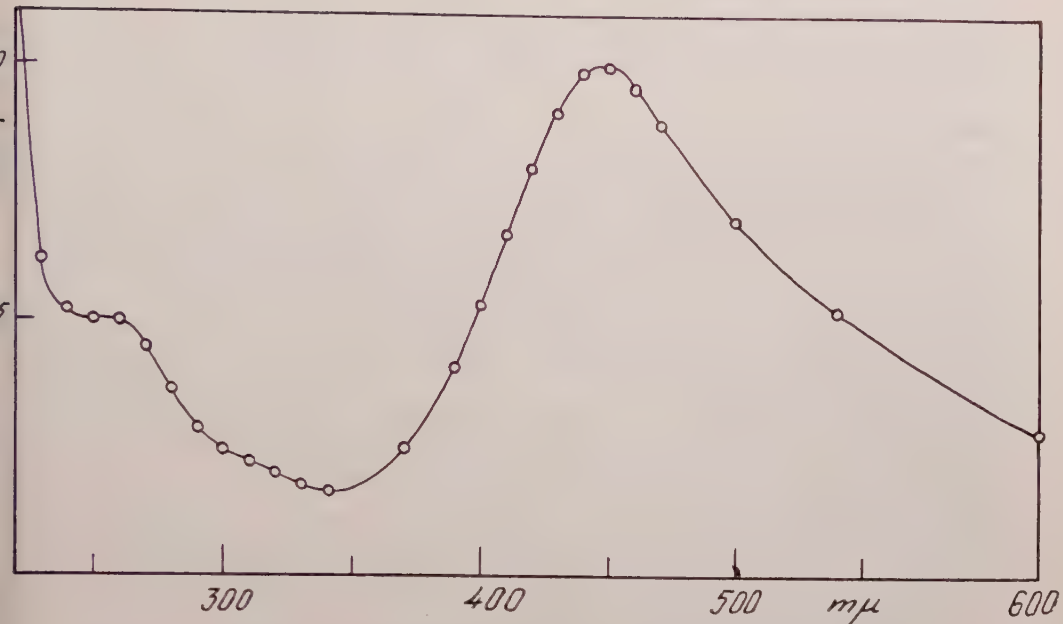


Fig. 1. Brenztraubensäure-2,4-Dinitro-phenylhydrazon.
Konzentration: 14,4 mg/100 ml 0,8 n. NaOH.

Flüssigkeit und dann ebensoviel physiol. NaCl-Lösung der zu untersuchenden wachstumshemmenden Substanz auf und führen die Mischung sofort in das Versuchstier intraperitoneal ein.

Oder wir nehmen die Mischung der Ascites-Flüssigkeit mit steigenden Mengen der cancerostatischen Substanz vor und injizieren diese Mischungen in eine Serie von Versuchstieren.

Für die mikroskopischen Untersuchungen der Sarkomzellen wurden Abstrichpräparate hergestellt, an der Luft getrocknet und mit Giemsa-Lösung gefärbt.

Zur Isolierung der Kerne wurden Zellen mit 1 %iger Zitronensäurelösung behandelt (vgl. Euler, Inga Fischer, Arkiv f. Kemi 22 A, nr 4. — Euler u. Mitarb., Sv. Kem. Tidskr. 57, 217 (1945). G. Klein (l. c.) hat an Ehrlich-Ascites-Sarkom mit dieser Methode gute Resultate erhalten.

Methodik der BTS-Bestimmung

Bezüglich dieser Methode nach dem von Case[1] und von Lu[2] angewandten Prinzip kann auf die Arbeiten von Euler, Fr. Schlenk und B. Högberg[3] verwiesen werden.

Wir haben die Bestimmungen des Dinitrophenylhydrazons teils, wie früher, im Stufenphotometer vorgenommen, und teils im Beckman-Apparat, was neben der grösseren Genauigkeit der Messungen den Vorteil hat, dass mit kleineren Mengen gearbeitet werden kann.

Für die vorliegenden und folgenden Versuche wurde eine Standard-Kurve der Absorption ausgearbeitet (Fig. 1).

---

[1] Case, Biochem. Jl. 26, 753 (1932).

[2] Lu, Biochem. Jl. 33, 249 (1939). Siehe auch: G. A. Le Page, Cancer Res. 10, 393 (1950).

[3] Euler, Fr. Schlenk u. B. Högberg, Uppsala Läkarefören. Förh. 45, 301 (1939).

Papierchromatographie des Yoshida-Ascites-Sarkomes[1]

Papier: Munktell OB

Chromatogramm A

Lösung: 4 Tle. n-Butanol, 1 Tl. Eisessig, 1 Tl. Wasser.

Entwicklung durch Ninhydrinlösung.

I. Festes Sarkom: 2 g wurden drei Stunden lang in 100 ml 1 %iger HCl-Lösung bei 100° hydrolysiert. Dann wurde die Reaktionslösung im Vacuum bis auf 5 ml eingeengt und filtriert. Die klare Lösung wurde nach Neutralisation zur Chromatographie verwendet.

II. Ascites-Flüssigkeit: 10 ml wurden mit so viel HCl versetzt, dass die Koncentration 1 %ig wurde; Hydrolyse wie unter I. Die Lösung wird im Vacuum auf 5 ml eingeengt und neutralisiert.

Die Chromatogramme von I und II lassen bezüglich Aminosäuren keinen Unterschied erkennen zwischen dem festen Gewebe und den Ascites-Zellen. Die Anzahl der Aminosäuren kann auf wenigstens 16 geschätzt werden.

Chromatogramm B

Die Ascites-Flüssigkeit wurde im Vacuum bei 40° auf $^1/_3$ des ursprünglichen Volumens eingeengt, von wenig festen Substanzen durch Zentrifugieren befreit und zum Chromatographieren angesetzt.

Der Vergleich des Chromatogramms B mit dem Chromatogramm A zeigt, dass die Anzahl freier Aminosäuren in der Ascites-Flüssigkeit abgenommen hat.

Chromatogramm C

Lösung: 77 Tle. n-Butanol, 13 Tle. Wasser, 10 Tle. Ameisensäure.

Entwicklung: Soda-alkalische diazotierte Sulfanilsäure-Lösung (DSS).

Vor der Bespritzung wurde das Papier im UV-Licht beobachtet.

2 Flecken: a) rot, Rf = 0 (fluoresziert). — b) gelb, Rf 0,165.

Papierchromatographie der mit Alkohol behandelten Ascites-Flüssigkeit

Methodik: siehe oben. Lösung wie Chromatogramm A.

Entwicklung durch Ninhydrinlösung und durch diazotierte Sulfanilsäurelösung.

Ascites Nr. 31: 2,5 ml Ascites-Flüssigkeit wurden mit 7,5 ml 96 %igem Alkohol versetzt, wodurch eine Fällung entsteht, in welche die Substanz eingeht, die mit DSS im Chromatogramm C Rotfärbung zeigt ($R_f = 0$). Die Fällung wurde abcentrifugiert und mit etwas Alkohol gewaschen. Die Alkohol-Lösung wurde im Vacuum bis auf etwa 2 ml eingeengt, wodurch eine weitere Fällung entstand. Die nach Abzentrifugieren derselben klare Lösung wurde chromatographiert. Zwei Flecken wanderten gleichzeitig; der eine Streifen wurde mit Ninhydrin, der andere mit DSS entwickelt.

Aus dem Chromatogramm ergibt sich, dass ca. 14 verschiedene Aminosäuren sich im Alkohol-Extrakt befanden. Bemerkenswert ist dass die Aminosäuren mit R_f-Werten zwischen 0,35 und 0,75 zu fehlen scheinen, unter diesen Alanin und Methionin, vielleicht auch α-Amino-buttersäure und Phenylalanin.

Anwesend sind Cystin und Cystein.

---

[1] Im Folgenden werden nur die Hauptergebnisse der chromatographischen Versuche kurz erwähnt. Die Chromatogramme selbst werden an anderer Stelle mitgeteilt.

Der mit DSS entwickelte Streifen enthält zwei gelbe Flecken von unbekannter Herstammung mit $R_f = 0{,}12$ und $0{,}29$, ferner einen schwächeren Flecken mit $R_f = 0$, offenbar der gleiche, der im Chromatogramm C auftrat.

Ascites-Flüssigkeit: Die gleiche wie zum Chromatogramm A verwendet. Hefenucleinsäure wurde mit 1 %iger Salzsäure hydrolysiert. Im UV-Licht erschien bei der Ascites-Flüssigkeit ein bläulich-fluorescierender Flecken mit $R_f = 0$. Der gleiche Flecken wurde nach Bespritzung mit diazotierter Sulfanilsäure intensiv rot, und gleichzeitig wurde ein zitronengelber Flecken mit $R_f = 0{,}165$ erhalten. Keiner dieser Flecken war identisch mit den durch hydrolisierte Nucleinsäure erhaltenen. Adenin trat im UV-Licht nicht hervor, und zwar wegen der erheblichen Eigenfluorescenz des verwendeten Papiers im blau-violetten Gebiet. Die hydrolysierte Ascites-Flüssigkeit enthält keine Stoffe, welche aus der Nucleinsäure durch Hydrolyse entstehen.

Yoshida-Ascites-Sarkom. Die Ascites-Flüssigkeit

Aus der stark trüben milchigen Flüssigkeit scheidet sich beim Stehen in wenigen Minuten, vollständiger bei Zentrifugieren, eine weissliche Masse ab. Das mit Giemsa-Lösung gefärbte Abstrich-Präparat zeigt im Mikroskop nur gleich grosse, gleich gefärbte runde Zellen.

Das gesamte Trockengewicht (erhalten durch Eindunsten der Ascites-Flüssigkeit bei 90°) schwankte zwischen 6 % und 8,5 %.

Die Gesamttrockensubstanz enthält etwa 36 % Zellen.

Versetzt man die Ascites-Flüssigkeit mit der dreifachen Menge Äthylalkohol, so entsteht eine Fällung, und zwar beträgt dieselbe rund 4 % der Ascites-Flüssigkeit.

Die übrigbleibende Flüssigkeit enthält noch etwa 0,8 % Aminosäuren und andere wasserlösliche Substanzen.

Das gesamte Trockengewicht der Lösung A, erhalten durch Eindunsten, betrug 3 %. Wir geben einen Versuch an:

20 ml Ascitesflüssigkeit wurden zentrifugiert und die überstehende klare Lösung A wurde abdekantiert. Das Zentrifugat (die Sarkomzellen) wurde mit physiologischer Kochsalz-Lösung gewaschen und bei 90° getrocknet. Trockengew. variiert 1–3 %.

Fällung von Lösung A mit Trichloressigsäure: 2 ml Lösung A wurden mit 5 ml 10 %iger Trichloressigsäure-Lösung versetzt. Die Fällung, hauptsächlich Eiweisskörper (einschliesslich Nukleoproteide), wurde abzentrifugiert, gewaschen, bei 90° getrocknet. Das Trockengewicht betrug ca 0,09 g = 4,5 %.

Lösung A wurde auch im Beckman-Apparat gemessen. Die im Verhältnis 1:40 verdünnte Lösung A ergab die Extinktionskurve der Fig. 2. Maximum bei 278 mμ entspr. dem für Aminosäuren im Protein gefundenen.

Über die Haltbarkeit bzw. Lebensdauer von Ascites-Tumorzellen liegen bereits Angaben vor. In Übereinstimmung mit diesen finden wir eine bemerkenswerte Stabilität der Yoshida-Ascites-Sarkomzellen. Bei einer Normaltemperatur von etwa 20° geht die Wirksamkeit dieser Sarkomzellen innerhalb 24 St. um etwa $^1/_3$ zurück.

Auf Grund früherer Versuche über den Einfluss von Röntgenstrahlen auf das Jensen-Sarkom haben wir auch den gleichen Einfluss auf die Yoshida-Ascites-Sarkomzellen untersucht und zwar in der dem lebenden Tier entnommen Ascites-Flüssigkeit. In den bis jetzt ausgeführten Versuchen mit Bestrahlung bis zu 2500 r wurde eine Abnahme auf rund 5 % konstatiert.[1]

---

[1] Herrn Laborator Dr. A. FORSSBERG sie auch hier bestens gedankt.

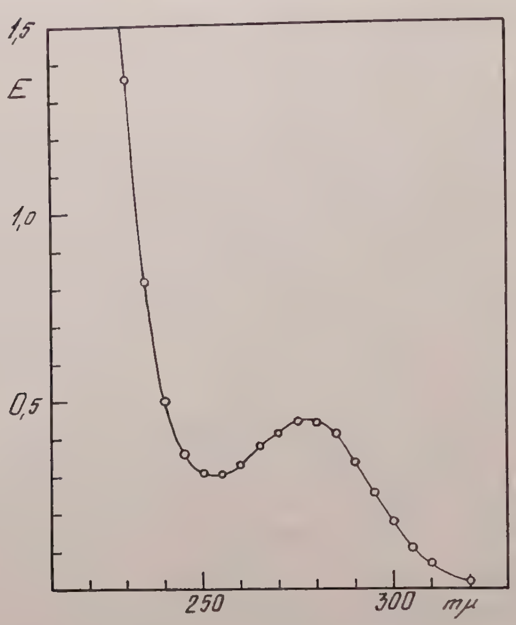


Fig. 2. Yoshida-Ascites-Flüssigkeit verdünnt 1 : 40.

Brenztraubensäure-Gehalt des Blutes von Ascites-Sarkom-Ratten

In einer Untersuchung von Euler und B. Högberg[1] hatte sich das Resultat ergeben, dass die Brenztraubensäure (BTS)-Konzentration des Blutes von Jensensarkom-tragenden Ratten das 1,6–3,8fache des BTS-Gehaltes des normalen Rattenblutes beträgt[2]. Dieser von anderen Forschern, u. a. von H. W. Schmidt[3] bestätigte Befund ist nicht auf das Jensen-Sarkom beschränkt; den Einfluss verschiedener anderer Tumorarten auf den BTS-Gehalt des Blutes haben Euler, L. Ahlström, I. Rönnestam-Säberg und I. Pettersson[2] in einer Tabelle zusammengestellt, der wir die folgenden Zahlen entnehmen.

Tumorart	Versuchstier	γ BTS/ml Blut	Rel. Erhöhung
Kein Tumor	Ratten	12–15	—
Jensen-Sarkom	Ratten	30–50	2,3–3,8
Brown-Pearce-Carcinom	Kaninchen	etwa 40	1,6–2,0
Ehrlich-Carcinom	Maus	28	1,6
Chemotumoren nach Inj.	Ratten	23	1,8
Transplant. Chemotumoren	Ratten	30	2,3

---

[1] Euler u. B. Högberg, Arkiv f. Kemi etc. 13 B, nr 16 (1939); 14 B, nr 29 (1940). — B. Högberg u. Fr. Schlenk, Arkiv f. Kemi etc. 14 B, nr 4 (1940).

[2] Euler, L. Ahlström, I. Rönnestam-Säberg u. I. Pettersson, Z. Krebsforsch. 55,1 (1944). — Im Blut menschlicher Krebspatienten ist die BTS-Erhöhung gering; vgl. Euler, Fr. Schlenk u. B. Högberg, Uppsala Läkarfören. Förh. N. F. 14, 301 (1939).

[3] H. W. Schmidt, Z. Krebsforsch. 54, 170 (1943); 51, 340 (1941); Biochem. Z. 312, 1 (1942).

An Mäusen trat durch ein Lymphosarkom keine BTS-Erhöhung im Blut ein.

Die Brenztraubensäure, die bekanntlich eine Funktion des Aneurin- bzw. des Co-Carboxylase-Gehaltes[1] des Organismus ist, nimmt im tierischen Stoffwechsel eine zentrale Stellung ein: Sie wird einerseits durch Milchsäuredehydrase unter Mitwirkung der Cozymase bis zu einem Gleichgewicht zu Milchsäure dehydriert. Andererseits wird BTS oxydativ abgebaut, und zwar tritt eine enzymatische Abspaltung von CO_2 ein. Die durch das Co-Enzym A aktivierte Essigsäure kann sich dann mit Oxalessigsäure zu Zitronensäure verbinden.

Neben diesem, für die Milch- und Brenztraubensäure-Bildung, überhaupt für den Zitronensäure-Cyklus seit Krebs[2] und Martius[3] angenommenen Reaktionsablauf findet, wie man nunmehr weiss, auch eine *unvollständige Oxydation* der Glucose statt, bei welcher die Zwischenprodukte des Reaktionscyklus nur teilweise unter CO_2-Verlust aufoxydiert werden.

Bekanntlich ist der Kohlehydratumsatz im Krebsgewebe auffallend hoch, woran die in Tumoren besonders aktivierte *Adenosin-triphosphorsäure* beteiligt ist. Gerade für den Tumor ist nun die unvollständige Oxydation kennzeichnend, bei welcher die Brenztraubensäure sowie die Zwischenprodukte des Zitronensäure-Cyklus für die Proteinsynthese zur Verfügung stehen. Sie gehen über Iminosäuren in Aminosäuren über (Brenztraubensäure in Alanin, α-Ketoglutansäure in Glutaminsäure), die selbst oder nach Umaminierung[4] der Kondensation zu Peptidketten unterliegen und so die intensive Proteinsynthese der Tumoren ermöglichen. Als spezifischer Antagonist der ATP scheint Colchicin zu fungieren.

Die Entwicklung des Yoshida-Ascites-Sarkoms verläuft, wie erwähnt, ausserordentlich schnell und führt nach 8–10 Tagen zum Tod der Tiere. Es war deshalb von Interesse zu untersuchen, welchen Einfluss dieser so aktive Tumor auf den Stoffwechsel und zwar zunächst auf den BTS-Gehalt des Blutes hervorruft.

Neue BTS-Versuche

Wir mussten zuerst den *normalen* BTS-Gehalt des Blutes der beiden verwendeten Rattenstämme feststellen.[5] Für unseren Albino-Stamm hatten wir früher aus hunderten von gut übereinstimmenden Messungen den Mittelwert gefunden:

$$12\text{–}13\ \gamma\ \text{BTS/ml Blut.}$$

Annähernd der gleiche Mittelwert (13–14 γ BTS) ergab sich nun sowohl für unseren Albino-Stamm, wie auch für den gelben Rattenstamm BD I.

Nach Einimpfung frischer Ascites-Sarkom-Flüssigkeit (unmittelbar nach der Entnahme aus dem Sarkom-Geber) erhielten wir die folgenden Werte (Tab. 1):

---

[1] R. A. Peters et al., Biochem. J. 24, 1872 (1933). — Der BTS-Gehalt des Blutes wird auch stark hormonal beeinflusst, besonders durch Insulin und Adrenalin, sowie durch Sexualhormone: Euler, I. Säberg, B. Högberg (Klin. Wochschr. 19, 433 (1940): G. Nylin, Euler u. B. Högberg).

[2] Krebs, Enzymologie 4, 148 (1937); Biochem. J. 31, 772 (1937). — F. Lipmann, Skand. Arch. Physiol. 76, 255 (1937); Naturwiss. 143, 436 (1939).

[3] C. Martius, Z. physiol. Chem. 247, 104 (1937). — Euler, E. Adler, G. Günther u. N. B. Das, Z. physiol. Chem. 254, 61 (1938). — Euler, I. Säberg, B. v. Euler, B. Högberg, Z. physiol. Chem. 268, 171 (1941); 270, 125 (1941).

[4] E. Adler, Euler, G. Günther and M. Plass, Biochem. J. 33, 1028 (1939).

[5] Die Blutentnahme geschah wie früher aus dem dekapitierten Tier; das Blut wurde sofort mit Heparin versetzt.

Tabelle 1.

Albino-Ratten		Bemerkung	Tage nach Impfung	γ BTS/ml Blut
Geschl.	Nr.			
♂	31		2	15,2
♂	32		5	19,3
♂	30		8	24,3
♂	48		7	25,5
♂	47	Nach 2 mg ATP	8	14,3
♂	39	Neg. gegen Jensen. Keine Ascites-Fl. Nur Spur von Tumor	2	20,5
♂	36	Neg. Jensen	5	27,5

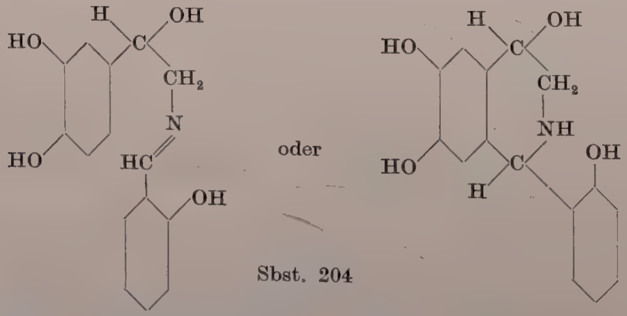
Die Ratten Nr. 39 und 36 waren mehrere Monate vor diesem Versuch mit Jensen-Sarkom ohne Erfolg geimpft worden.

Wir haben nun begonnen, diese Yoshida-Ascites-Ratten zur Untersuchung der cancerostatischen Wirksamkeit verschiedener Stoffe zu verwenden, die wir gleichzeitig mit der Ascites-Flüssigkeit eingeimpft haben. Die Tabelle 2 enthält einige Angaben über den BTS-Gehalt des Blutes.

Tabelle 2.

Albino-Ratten	Tage nach Impfung von 0,3 ml Ascites-Flüss.	Gleichzeitige Einführ. von	γ BTS per ml Blut
♂ 45	7	2 mg Sbst. 204	20,6
♂ 46	8	„ „ „ „	
♀ 51	4	1 mg Adrenochrom	16
♀ 52			
♀ 53	7	0,5 mg Colchicin[1]	19
♀ 54	6	0,5 » » + ATP	24
♀ 57	5	50 γ Adrenalin	29
♀ 60	7	200 γ Arterenol	33

Die Sbst. HH 204, $C_{15}H_{15}O_4N$, wurde erhalten aus L-Arterenol (L-Nor-Adrenalin) und Salicylaldehyd als Beispiel eines Kondensationsproduktes von Arterenol mit einem Aldehyd.



Sbst. 204

---

[1] Blutbild: Dixon u. Malden, J. Physiol 37, 50 (1908).

Adrenochrom hatte sich bei Injektion in Jensensarkom-tragende Ratten als cancerostatisch unwirksam erwiesen. Die Sbst. 204 soll in Mengen von 1 mg täglich weiter untersucht werden, in Rücksicht auf den Befund von LETTRÉ über die mitostatische Wirkung dieses Adrenalin-Derivates.

Zum Vergleich wurde auch die Einwirkung von L-Adrenalin selbst an dem Yoshida-Ascites-Sarkom untersucht und sehr klein befunden.

Einfluss des Yoshida-Ascites-Sarkomes auf die prozentische Zahl der vitalfärbbaren roten Blutzellen

In den Jahren 1936–1939 wurde in diesem Institut an Meerschweinchen die Zahl der vitalfärbbaren roten Blutzellen (Reticulozyten) untersucht, um ihre Abhängigkeit von hormonalen und diätischen Einflüssen festzustellen. Veranlasst durch die dabei gewonnenen Ergebnisse wurden die gleichen Einflüsse auf das rote Blutbild unserer Albino-Ratten studiert[1], besonders der Zusammenhang zwischen der Reticulozytenzahl und dem Mangel der Diät an Vitamin A und an Vitaminen der B-Gruppe. Dabei ergab sich eine Beziehung zwischen der rel. Reticulozytenzahl und dem Wachstum (der Gewichtszunahme) der Versuchstiere.

Im Anschluss an diese Versuche hat NORA BARGONI dem roten Blutbild der Ratten ebenfalls ihre Aufmerksamkeit gewidmet; sie untersuchte die Reticulozytenzahl (rel. zur Gesammtmenge der roten Blutkörperchen) zunächst an Tieren, die unter Vitamin-A-Mangel ernährt wurden, dann aber besonders an cancerösen, Jensensarkome tragenden Ratten. Bei dieser Untersuchung wurde gleichzeitig der Brenztraubensäure-Gehalt des Blutes bestimmt. NORA BARGONI[2] fasst ihre Resultate in folgender Tabelle 3 zusammen:

Tabelle 3.

Versuchsbedingung	Konz. der BTS per ml Blut	Rel. Zahl der Reticulozyten	Katalase-Wirk. der Leber
Mangel an Vit. A	Erhöhung	Erniedrigung	
Einfluss d. Sarkomes	Erhöhung	Erniedrigung	Erniedrigung

Die Reticulozytenzahl der normalen Ratten hatte unter den damals eingehaltenen diätischen und sonstigen Versuchsbedingungen den auffallend geringen Wert 1,43 % (Erythrozyten in mm^3 Blut $= 6{,}5 \cdot 10^6$). Gleichzeitig angestellte Zählungen der Reticulozyten an Ratten, welche Jensensarkome von im Mittel 8 g trugen, ergaben den Wert 0,87 % (Erythrozyten in mm^3 Blut $= 6{,}1 \cdot 10^6$). Somit hatte das Sarkom eine Abnahme der Reticulozyten auf rund $^2/_3$ des ursprunglichen Wertes verursacht.

Analoge Versuche wurden an unseren BD I-Ratten angestellt. (Zur Versuchsmethodik siehe EULER u. M. MALMBERG, Z. physiol. Chem. 243, 121 u. 249, 85; 1936/37.)

Die mittlere Reticulozytenzahl der normalen männlichen BD I-Ratten betrug ca 1 % (Erythrozytenzahl: $8{,}5 \cdot 10^6$ mm^3 Blut). Drei Tage nach der Injektion von 0,5 ml

---

[1] EULER u. M. MALMBERG, Z. physiol. Chem. 261, 103 (1939); Naturwiss. 27, 166 (1939).

[2] N. BARGONI, Dieses Archiv 1, nr 17 (1949).

Yoshida-Ascites-Flüssigkeit erhielten wir im Mittel 2 % vitalfärbbare rote Blutzellen. An den gleichen Ratten betrug der BTS-Gehalt des Blutes

Normal 14 γ/ml; nach Ascites-Injektion 30 γ/ml.

Der Einfluss des Yoshida-Ascites-Sarkomes auf die Reticulozyten-Zahl zeigte sich also im wesentlichen gleich dem früher mit Jensensarkom erhaltenen Effekt.

Herrn Ingenieur G. Hanshoff danken wir für seine Mitarbeit.

Stockholm, Universität, Vitamin-Institut und Institut für organ.-chemische Forschung.

Tryckt den 24 maj 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 30

Communicated 27 January 1954 by ARNE FREDGA

On the preparation and Grignard reaction of 3-bromothiophene

By SALO GRONOWITZ

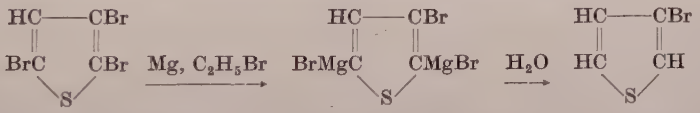
With 1 figure in the text

Owing to the reactivity of the 2-positions of thiophene it is difficult to prepare 3-substituted thiophenes with unblocked 2-positions. Before CAMPAIGNE and LESUER (1) worked out the side-chain bromination of 3-methylthiophene only a few 3-substituted derivatives of thiophene had been prepared through 3-halothiophenes.

RINKES (2) has synthesized 3-iodothiophene in a two-step synthesis over tetraiodothiophene. This method, however, is very expensive and difficult and the reduction of tetraiodothiophene can, owing to its limited solubility, only be carried out in small portions.[1]

The best method of preparation of 3-bromothiophene has hitherto been a five-step synthesis over 2-thiophenecarboxylic acid worked out by STEINKOPF (3). Recently HURD and ANDERSON (4) have prepared 3-bromothiophene through direct bromination of thiophene in the vapour phase at high temperatures. On account of the low yield this method is only of theoretical interest.

STEINKOPF *et al.* (5) have attempted to prepare 3-bromothiophene by hydrolysing the Grignard reagent formed from 2,3,5-tribromothiophene by the method of "entrainment" using methyl bromide as the auxiliary halogenide.



However, their yields were so poor that they abandoned this method and worked out the above-mentioned five-step synthesis of 3-bromothiophene. Recently TROYANOWSKY (7) mentions briefly that he has obtained a better yield using the method of STEINKOPF *et al.*

Because of the high price and difficult availability of 3-methylthiophene, and the highly lachrymatory and vesicant properties of 3-thenylbromide and its strong tendency of polymerisation, the present author has tried to find a better method for the preparation of 3-bromothiophene in order to use it as an intermediate in the preparation of 3-substituted derivatives of thiophene.

---

[1] The statement of HARTOUGH (6), that 3-iodothiophene in contrast to 3-bromothiophene forms the Grignard reagent in high yields, seems to be due to some misunderstanding. Both substances seem to undergo the Grignard reaction only by aid of the "entrainment" method.

It was found that it is possible to obtain 3-bromothiophene in very good yield by STEINKOPF's method using large amounts of ethyl bromide and longer time of reaction. The yields of 3-bromothiophene depend strongly upon the amounts of ethyl bromide used. (Table 1.)

Table 1.

2,3,5-tribromothiophene, g	Ethyl bromide, g	3-bromothiophene, g	Yield, %	2,3- and 2,4-dibromothiophene, g	Yield, %
141 (0.44 moles)	54.5 (0.5 moles)	4	5.6	85	80
160 (0.5 moles)	109 (1.0 moles)	27	33	51	42
576 (1.8 moles)	590 (5.4 moles)	150	51	105	24

Contrary to STEINKOPF *et al.* it was found that the mixture of 2,3-dibromothiophene and 2,4-dibromothiophene could, in the same way, be almost quantitatively converted to 3-bromothiophene.

The infra-red spectrum of 3-bromothiophene which as far as the author knows has not been published previously, shows an extremely strong band at 13.1 μ, which seems to be typical for 3-substituted thiophenes, and which has earlier been found in 3-methylthiophene and 3-thiothienol (3-mercaptothiophene) (8). 2-Bromothiophene shows a sharp maximum at 10.26 μ, which seems to be almost absent in 3-bromothiophene. It may be possible to use this maximum to determine the composition of mixtures of 2-bromothiophene and 3-bromothiophene. For comparison the spectrum of 2-bromothiophene is shown. It is identical with that previously published by HOCHGESANG (9).

3-Bromothiophene readily undergoes the Grignard reaction, if large amounts of an auxiliary halogenide such as ethyl bromide are used. The yield of the Grignard reagent was determined through subsequent carbonation and isolation of 3-thiophenecarboxylic acid. The yield of 3-thiophenecarboxylic acid was increased from 20 % to 56 % by varying the proportion of 3-bromothiophene to ethyl bromide from 1:1 to 1:5.

Both 2,3,5-tribromothiophene and 3-bromothiophene seem to belong to that group of halogen compounds which undergo the Grignard reaction according to the "entrainment" method. This modification of the Grignard reaction was first worked out by GRIGNARD (10) himself and good results were obtained with substances giving an insoluble magnesium bromide. GRIGNARD explained the effect of the auxiliary halogenide by the assumption that it kept the magnesia in an activated state and that a more soluble bimolecular complex was formed. The statement of CLÉMENT (11) that the best yields were obtained when exactly equimolar amounts of ethyl bromide were used could not be confirmed by the present author, but the solubility properties seem to be of some importance. In the Grignard reaction of 2,3,5-bromothiophene two liquid phases were obtained, when one or two moles of ethyl bromide were used, but only one phase when the proportion between 2,3,5-tribromothiophene and ethyl bromide was 1:3.

HARTOUGH (12) has proposed that it should be a double conversion:

$$\mathrm{RMgBr} + \mathrm{R'Br} \longrightarrow \mathrm{R'MgBr} + \mathrm{RBr}.$$

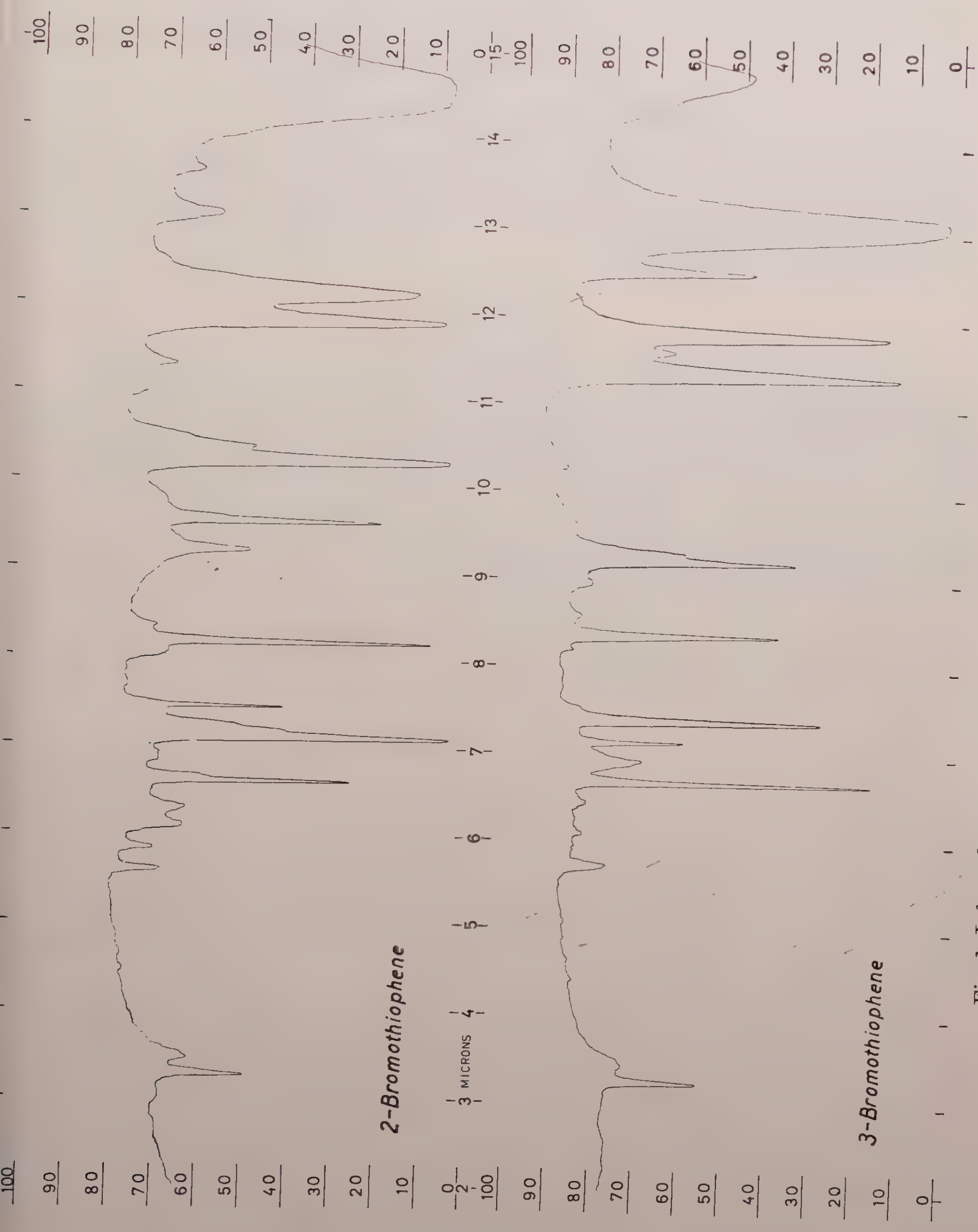


Fig. 1. Infra-red spectra of 2-bromothiophene and 3-bromothiophene.

This mechanism was first suggested by Urion (13) to explain the "entrainment" reaction, but it was found that a solution of ethyl magnesium bromide, free of metallic magnesium, only gave traces of 3-thiophenecarboxylic acid with 3-bromothiophene. It seems that the halogen-metal interconversion reaction is not of great importance in the case of Grignard compounds. The author has found, however, that 3-bromothiophene smoothly gives such interconversion with *n*-butyllithium (14).

This method of synthesizing 3-thiophenecarboxylic acid is of course more time-consuming than the method of Campaigne and LeSuer (15) recently published in *Organic Synthesis*, but the yields are comparable and it has the advantage of starting with cheap and easily available chemicals.

Experimental

3-Bromothiophene. 230 g of magnesium turnings in the usual Grignard apparatus were covered with one litre of absolute ether. The reaction was started with some pure ethyl bromide, and a mixture of 590 g (5.4 moles) of ethyl bromide and 576 g (1.8 moles) of 2,3,5-tribromothiophene (16) in two litres of absolute ether were added at such a rate, that the reaction mixture refluxed gently. The addition was complete after nine hours and the mixture was refluxed for an additional forty hours. The reaction mixture was then hydrolysed by pouring it into a mixture of dilute sulphuric acid and ice. The ether layer was separated and the aqueous layer extracted with ether. The organic layer was dried, the ether distilled off and the residue fractionated. The yield of 3-bromothiophene boiling at 156°–159° was 150 g (51 %) and of 2,3-dibromothiophene and 2,4-dibromothiophene 105 g (24 %) boiling at 84°–86°/10 mm Hg.

The experiments in which smaller amounts of ethyl bromide were used were carried out in the same way.

3-Bromothiophene from the dibromide mixture. 15.8 g (0.65 moles) of magnesium turnings were covered with 100 ml of absolute ether. The reaction was started with some ethyl bromide and a mixture of 75 g (0.31 moles) of the dibromide mixture obtained above, and 35 g (0.32 moles) of ethyl bromide in 500 ml absolute ether were added at such a rate that gentle reflux was obtained. The reaction mixture was refluxed for 24 hours and worked up as above. 40 g (80 %) of 3-bromothiophene boiling at 156°–159° were obtained.

3-Thiophenecarboxylic acid. 7.3 g (0.3 moles) of magnesium turnings were covered with 50 ml of absolute ether. The reaction was started with a little ethyl bromide and a mixture of 8.1 g (0.05 moles) of 3-bromothiophene and 27.5 g (0.25 moles) of ethyl bromide in 150 ml of absolute ether was added at such a rate that gentle reflux was obtained. The mixture was refluxed for 36 hours and poured on a slurry of dry ice and ether. When the dry ice had evaporated the mixture was hydrolysed with dilute sulphuric acid. The ether layer was separated, and the aqueous phase several times extracted with ether. The combined ether layers were extracted several times with 2-*N* sodium hydroxide. The aqueous layer was acidified in the cold with 1:1 hydrochloric acid precipitating 3.3 g of 3-thiophenecarboxylic acid. Extracting the mother liquor with ether and evaporating the ether gave an additional 0.3 g, making a total yield of 56 %. Recrystallized from hot water and decolorized with charcoal the pure white acid melted at 137.5°–138.5°.

The experiments in which smaller amounts of ethyl bromide were used were carried out as above. Using 3-bromothiophene and ethyl bromide in proportions of 1:1 and 1:3 respectively the corresponding yields were 20 % and 42 %.

Infra-red spectra

The 3-bromothiophene used was distilled through a column having 50 theoretical plates. The infra-red spectra were recorded with a Perkin-Elmer mod. 21 spectrophotometer equipped with NaCl prism. Both substances were examined as liquids between two NaCl plates.

Summary

The effect of ethyl bromide upon the Grignard reaction of 2,3,5-tribromothiophene and 3-bromothiophene has been investigated. Good yields of 3-bromothiophene have been obtained using three moles of ethyl bromide per mole 2,3,5-tribromothiophene. 3-Bromothiophene has thus become an easily available intermediate for the synthesis of 3-substituted derivatives of thiophene.

The author wishes to express his thanks to Professor ARNE FREDGA for valuable discussions. Thanks are also due to Mr. A. ROSENBERG for taking the infra-red spectra and to Mr. R. ÖHBERG for assistance in part of the experimental work.

Department of Organic Chemistry, Chemical Institute, University of Uppsala, January 1954.

REFERENCES

1. CAMPAIGNE, E., and LESUER, W. M., J. Am. Chem. Soc. *70*, 1555 (1948).
2. RINKES, I. J., Rec. trav. Chim. *53*, 648 (1934).
3. STEINKOPF, W., Ann. *543*, 128 (1940).
4. HURD, C. D., and ANDERSON, H. J., J. Am. Chem. Soc. *75*, 3517 (1953).
5. STEINKOPF, W., JACOB, H., and PENZ, H., Ann. *512*, 136 (1934).
6. HARTOUGH, H. D., Thiophene and its derivatives. New York 1952, p. 215.
7. TROYANOWSKY, C., Compt. rend. *232*, 2236 (1951).
8. HARTOUGH, H. D., Thiophene and its derivatives. New York 1952, p. 121.
9. —— *ibid.* p. 118.
10. GRIGNARD, V., Compt. rend. *198*, 625 (1934).
11. CLEMENT, H., Compt. rend. *198*, 665 (1934).
12. HARTOUGH, H. D., Thiophene and its derivatives. New York 1952, p. 443.
13. URION, E., Compt. rend. *198*, 1244 (1934).
14. GRONOWITZ, S. To be published.
15. CAMPAIGNE, E., and LESUER, W. M., Org. Synthesis *33*, 94 (1953).
16. ROSENBERG, J., Ber. *18*, 1773 (1885).

Tryckt den 24 maj 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 31

Mitgeteilt am 10. Februar 1954 von J. A. HEDVALL

Diffusion von radioaktivem Blei in Bleioxydationsschichten

Von ROLAND LINDNER und HALDUN N. TEREM

Mit 4 Figuren im Text

Im Laufe der Erforschung des Mechanismus von Festkörperreaktionen mittels radioaktiver Indikatoren (1) wurde die Oxydation von Metallen untersucht durch Messung der Diffusion von radioaktiven Isotopen des Metalls in Oxydschichten, die sich auf dem betreffenden Metall gebildet haben.

Im Falle des Zinkes sind die Ergebnisse solcher Messungen (2) mit dem Oxydationsmechanismus nach WAGNER (3) vereinbar; die Messungen am Blei werden im folgenden mitgeteilt.

Die Metalloxydation

Es wurden Scheibchen (von etwa 8 mm Durchmesser und 2 mm Höhe) analytisch reinen Bleies („Merck", silberfrei, 99,999 %) nach Reinigung und Ätzung in 20%iger Salpetersäure im Sauerstoffstrom bei verschiedenen Temperaturen oxydiert, und die Gewichtszunahme nach entsprechenden Zeitabschnitten festgestellt. Auch oberhalb des Schmelzpunktes von metallischem Blei (327 °C) hielt die Oxydhaut das Metall zusammen, wie das auch bei der Pulveroxydation anderer Metalle gefunden worden ist (4).

Die Gewichtszunahmen folgten im allgemeinen mit guter Genauigkeit einer parabolischen Zeitfunktion; die Ergebnisse sind neben denen der Literatur (6–11) in Fig. 1 in der Darstellung $\log k'$ (in $\mathrm{cm}^2\ \mathrm{sec}^{-1}) = f(1/T)$ wiedergegeben.

Es ergibt sich:

1) Unsere Werte für die Anlaufkonstante stimmen — mit Hinsicht auf die nicht identischen Ausgangsstoffe — befriedigend mit denen der Literatur überein.

2) Im Existenzbereich der Mennige Pb_3O_4 erhalten wir bei 515 °C einen verhältnismässig tiefen Wert (für die „erste Reaktionsparabel"), in Übereinstimmung mit GRUHL (7) sowie WEBER und BALDWIN (5). (Hier mag auch die $\beta - \alpha$ Umwandlung des PbO hineinspielen, wahrscheinlich über eine erleichterte Bildung von Pb_3O_4.)

3) Bei jenem Versuch zeigt sich eine ausgesprochene „Induktionsperiode" (wie dies auch bei der Oxydation verschiedener Metalle in Pulverform gefunden wurde [12]), in Übereinstimmung mit WEBER und BALDWIN (5), die im Existenzbereich der Mennige eine erheblich grössere Neigung der zweiten Reaktionsparabel gegenüber der ersten fanden, was sie mit der Bildung einer äusseren Pb_3O_4-Schicht erklären.

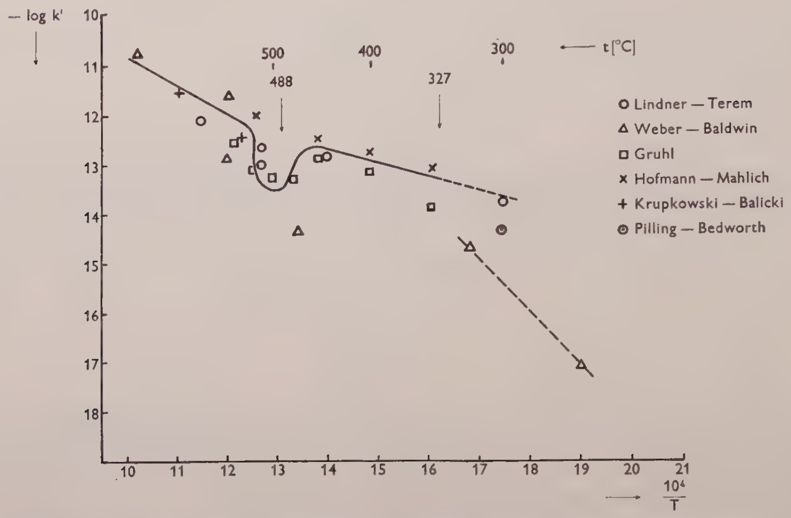


Fig. 1. Übersicht über die bisher vorliegenden Werte für die Oxydationskonstante des Bleis (in $cm^2 sec^{-1}$) als Funktion der Temperatur.

Die Selbstdiffusionsversuche

Die Diffusionskonstanten radioaktiven Bleis (ThB = Pb-212) wurden an den beschriebenen Oxydationstabletten nach der Hevesyschen Methode (13) des α-Rückstosses gemessen.

(Betreffs Einzelheiten der Methode vgl. eine frühere Veröffentlichung (14), gegenüber welcher als Verbesserungen die direkte ,,Exposition" der oxydbedeckten negativ geladenen Bleitabletten in der Thoriumemanation zwecks Herstellung einer dünnen Schicht ThB zu nennen ist, sowie die Verwendung eines ,,Counting-rate meters" mit registrierendem mA-meter zur abwechselnden Messung des ThC''-Zerfalles von Standard- und Diffusionspräparat.)

Die relative Rückstossausbeute als Funktion der Diffusionszeit bei den verschiedenen Temperaturen ist in Fig. 2 wiedergegeben. Die resultierenden Selbstdiffusionskonstanten sind in üblicher Weise in Fig. 3 dargestellt, welche als gestrichelte Linie auch den Verlauf für trägerfreies Bleioxyd enthält.

Die letzterem Verlauf zugrundeliegenden Werte entstammen alten Versuchen und sind für den Hochtemperaturzweig (Versuche in reinem Stickstoff) gesichert und veröffentlicht (15); für den Tieftemperaturzweig dagegen (eigene Versuche und Auswertung alter Angaben von Hevesy und Seith [16]) streuen sie stark.

Es zeigt sich eine befriedigende Übereinstimmung der Messungen ohne und mit Trägermetall, dessen Anwesenheit also die Diffusion nicht nennenswert beeinflusst. (In letztgenanntem Fall scheint im Versuch bei 520 °C eine diffusionsverlangsamende Wirkung des Luftsauerstoffes, analog der der Mennigebildung bei der Oxydation [s. o.] vorzuliegen, ein Befund, der allerdings durch weitere Versuche erhärtet werden müsste.)

Bei hohen Temperaturen sind die Messungen am ,,freien" Bleioxyd zuverlässiger, da hier die ,,Kontaktmethode" (14) angewendet werden kann; bei tieferen

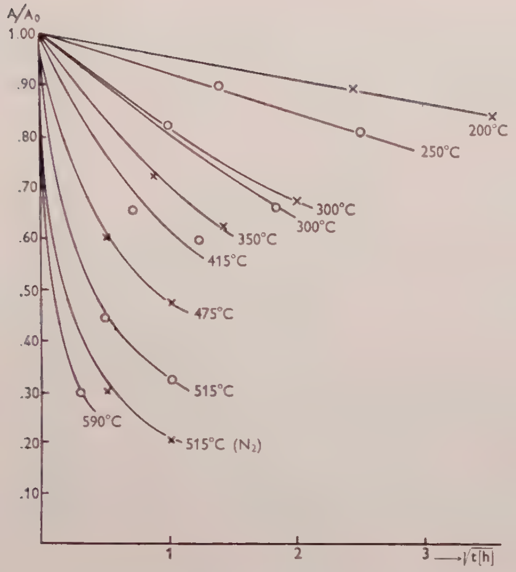


Fig. 2. Relative Rückstossausbeute als Funktion der Diffusionszeit bei verschiedenen Temperaturen.

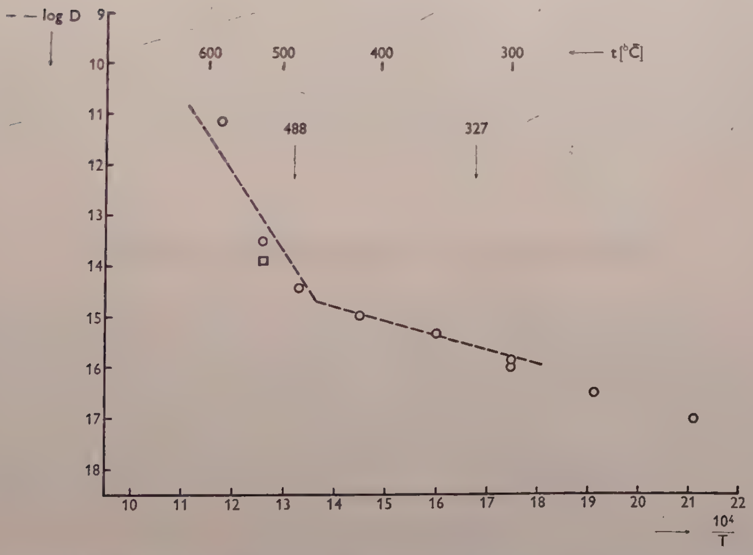


Fig. 3. Diffusionskonstante von Blei in Bleioxyd als Funktion der Temperatur, Messwerte für Oxydationsschichten; die gestrichelte Linie bezieht sich auf Oxydsintertabletten.

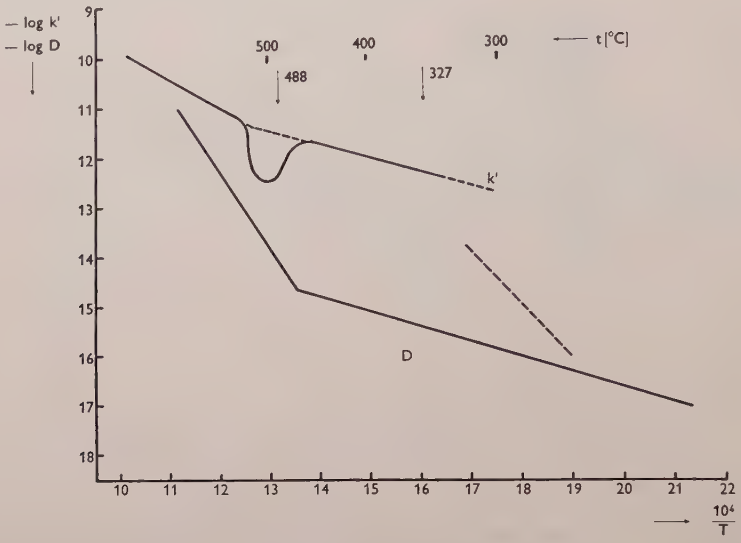


Fig. 4. Vergleich von Oxydationskonstante k' und Selbstdiffusionskonstante D (gestrichelte Linien: extrapolierter oder unsicherer Verlauf).

Temperaturen dagegen sind die Messungen an Bleioxyd auf Bleimetall vorzuziehen, da hier die radioaktive dünne Schicht offenbar mit besserer Reproduzierbarkeit durch direkte Exposition (s. o.) als durch die sonst übliche Aufdampfung aufgebracht wird.

Beide Messreihen zeigen in der Darstellung in Fig. 3 einen „Knick", der bei etwa 460 °C liegt. Dies könnte in Zusammenhang gebracht werden mit der kristallographischen Umwandlung des PbO, deren Gleichgewichtstemperatur allerdings zu 488 °C angenommen wird. Hiernach könnte man eine Aktivierungsenergie von 66 000 cal/mol für die Selbstdiffusion in α-PbO, eine solche von ca. 13 000 cal/mol für die in β-PbO annehmen.

Zusammenfassende Betrachtung der Ergebnisse

In Fig. 4 sind (in der gleichen Dimension) Oxydationskonstanten (Mittelwerte der bisher vorliegenden Untersuchungen, obere Kurve) und Selbstdiffusionskonstanten (mit Trägermetall) wiedergegeben. Es ist kaum möglich, einen Ausdruck für die Temperaturfunktion der Oxydationskonstante anzugeben. (Bei tieferen Temperaturen scheint sie von der Selbstdiffusion nicht sehr verschieden zu sein.)

Die Werte der Oxydationskonstante liegen erheblich (bis zu 3 Zehnerpotenzen) höher als die der Selbstdiffusion in einem weiten Temperaturintervall, in dem die Diffusion von Bleiionen in Bleioxyd nicht als bestimmend für die Geschwindigkeit der Bleioxydation angesehen werden kann.

Die Untersuchungen werden fortgesetzt, und eine nähere Betrachtung des Oxydationsvorganges soll demnächst gegeben werden.

Wir danken Prof. J. A. HEDVALL für sein Interesse an der Untersuchung sowie *Statens Tekniska Forskningsråd* und *Överläkare Albert Wallins Fond* für die Bereitstellung von Mitteln.

Abteilung „Kristallkinetik", Institut für Silikatforschung, Chalmers Technische Hochschule, Göteborg (Schweden).

LITERATUR

(1) LINDNER, R., Z. El. Chem. *54*, 430 (1950.)
(2) MOORE, W. J., J. Elektrochem. Soc. *100*, 302 (1953); LINDNER, R., 8. Skand. Chemiekongress, Oslo, Juni 1953.
(3) WAGNER, C., Z. Phys. Chem. (B) *21*, 25 (1953).
(4) TEREM, H. N., C. R. *224*, 1351 (1947).
(5) WEBER, E. und BALDWIN, W. M., Trans. AIME *194*, 854 (1952).
(6) HOFMAN, W. und MAHLICH, K. H., Werkstoffe u. Korrosion *2*, 55 (1951).
(7) GRUHL, W., Z. Metallkunde *40*, 225 (1949).
(8) BIRKUMSHAW, L. L. und PRESTON, G. D., Phil. Mag. *25*, 769 (1938).
(9) KRUPKOWSKI, A. und BALICKI, S., Hutnik *9*, 138 (1937).
(10) MCADAM, D. J. und GEIL, G. W., Bur. Stand. J. Research Wash. *28*, 593 (1942).
(11) PILLING, N. B. und BEDWORTH, R. J., J. Inst. Met. *29*, 529 (1923).
(12) TEREM, H. N., Bull. Soc. chim. (5) *6*, 664 (1939).
(13) DE HEVESY, G. und SEITH, W., Z. Physik *56*, 790 (1929).
(14) LINDNER, R., Acta Chem. Scand. *5*, 735 (1951).
(15) ——, K. Vet. Akad. (Stockholm) Arkiv f. Kemi *4*, Nr. 27 (1952).
(16) DE HEVESY, G. und SEITH, W., Ber. Naturforsch. Ges. Freiburg *28*, 2 (1928).

Tryckt den 24 maj 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 32

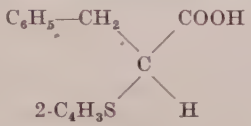
Communicated 27 January 1954 by Arne Fredga

The enanthiomorphs of α-(2-thienyl)hydrocinnamic acid and 2-thenylphenylacetic acid

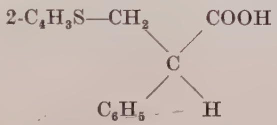
By Kurt Pettersson

With 4 figures in the text

In the course of a study of optically active 2-thiophene compounds (*1*), α-(2-thienyl)hydrocinnamic acid (I) and 2-thenylphenylacetic acid (II) have been synthesized (*1–2*). The racemic acids have now been resolved into optical antipodes and the melting-point diagrams determined.



(I)



(II)

The dextrorotatory α-(2-thienyl)hydrocinnamic acid is obtained by recrystallisation of the cinchonidine salt from dilute ethanol. The acid crystallises from petrol (b.p. 60°–70°) as colourless needles, melting at 71°–72°. The levorotatory acid is obtained from the *l*-ephedrine salt, purified by recrystallisation from dilute ethanol.

The optically active 2-thenylphenylacetic acids are obtained with the optically active α-phenylethylamines or 2-amino-1-phenylpropanes (benzedrines) (*3*) with dilute ethanol as solvent. The acids crystallise from petrol as colourless needles.

The enanthiomorphs are stable at their melting points and it was thus feasible to investigate the four possible two-component systems with a Kofler hot-stage microscope (*4*).

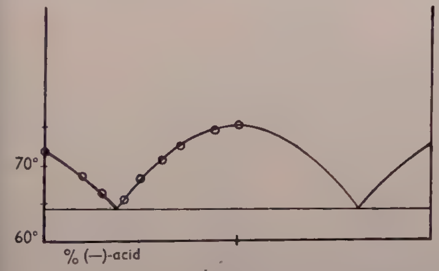


Fig. 1. (+)- and (−)-α-(2-Thienyl)hydrocinnamic acid.

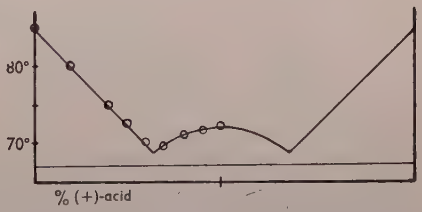


Fig. 2. (+)- and (−)-2-Thenylphenylacetic acid.

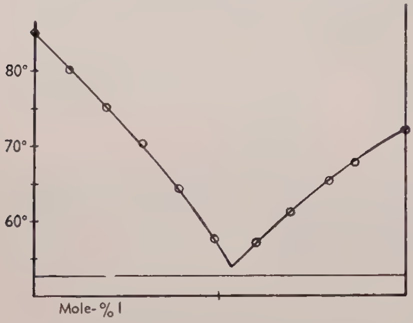


Fig. 3. (+)-2-Thienylhydrocinnamic acid (I)
(−)-2-Thenylphenylacetic acid (II).

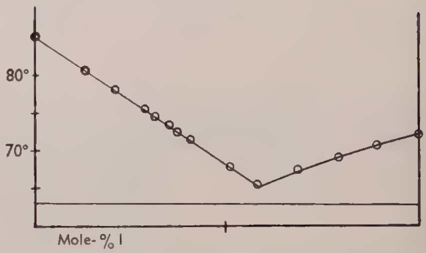


Fig. 4. (+)-2-Thienylhydrocinnamic acid (I)
(+)-2-Thenylphenylacetic acid (II).

It has often been pointed out that compounds, which differ in the sense that a phenyl group is replaced by a 2-thienyl group, may be able to form a quasi-racemate. However, such a formation is dependent also upon effects other than structural (*5–6*), and the tendency of the enanthiomorphs to form a true racemate seems to be of some importance. As is seen from the formulas (I) and (II), 2-thenylphenylacetic acid (II) is formally obtained from α-(2-thienyl)hydrocinnamic acid (benzyl-2-thienylacetic acid) (I) by a double interchange of a phenyl group for a 2-thienyl group. The structural differences may thus be greater than in the case of a single exchange. From Figures 1 and 2 it is also evident that 2-thienylhydrocinnamic acid has only a moderate tendency towards racemate formation, and for 2-thenylphenylacetic acid the corresponding tendency is rather low. Under such circumstances it is not surprising to find that the acids are not able to form a quasi-racemate (Figs. 3–4) and information about the steric relations between the acids cannot be obtained directly according to the quasi-racemate method. It is possible, however, that a connection between the two acids can be obtained through the structural intermediates α-phenylhydrocinnamic acid or 2-thenyl-2-thienylacetic acid (*7*).

In many cases information about the steric relations between similarly constituted compounds may be obtained by measuring the optical activity in a number of different solvents (*8*). Compounds belonging to the same steric series then show the same mode of variation of the optical activity. The molecular rotations of (+)-2-thienylhydrocinnamic acid [(+)-I] and of (−)-2-thenylphenylacetic acid [(−)-II] in representative solvents are collected in Table 1. The values afford a good foundation of the statement that the acids with opposite direction of rotation have also opposite configurations.

Table 1.

Solvent	Molecular rotation	
	(+)-I	(−)-II
Water (ion)	+156°	−211°
Benzene	+174°	−286°
Ethanol	+181°	−265°
Acetic acid	+185°	−278°
Acetone.	+205°	−297°

Experimental

Preliminary tests on resolution: About 0.001 moles of the acids I and II and equivalent amounts of the bases were dissolved in 10 ml of the hot solvents. The mixtures were allowed to stand at room temperature and some water was added gradually until crystals or oils were formed. The crystals were filtered off, the acid liberated and the optical activity measured in absolute ethanol (Table 2).

In some cases the compounds remained in solution even when a large excess of water was added. In the table these are indicated by s.

Table 2.

Base	$[\alpha]_D^{25}$ of acid in ethanol					
	benzene		ethanol		acetone	
	I	II	I	II	I	II
l-Ephedrine	−28°		−28°			±0°
Strychnine			−22°	+16°	+35°	−28°
Brucine			oil	oil	oil	oil
Quinine			−8°	−3°	−20°	+41°
Cinchonine			oil		oil	s
Cinchonidine			+45°	−19°		−15°
Nicotine			s	s		s
Yohimbine				oil		oil
(+)-α-Phenylethylamine			±0°	+61°		
(+)-Benzedrine	±0°	±0°	+17°	+70°		

Resolution of α-(2-thienyl)hydrocinnamic acid: 7.2 g (0.03 moles) of optically inactive benzyl-2-thienylacetic acid and 9.3 g (0.03 moles) of cinchonidine were dissolved in a hot mixture of 180 ml of ethanol and 150 ml of water. A few drops of the solution were set aside and allowed to crystallise and the principal part of the solution was seeded with the crystals formed. The solution was allowed to stand for 24 hours and the crystals were filtered off and dried. 0.5 g of the precipitate was set aside, the acid isolated and the optical activity measured in acetone. The remaining precipitate was recrystallised from dilute ethanol until the optical activity of the acid remained constant.

Crystallisation No.	1	2	3	4
ml of solvent (ethanol-water 6 : 5)	330	165	122	110
g of salt obtained	8.1	6.0	5.3	4.8
$[\alpha]_D^{25}$ of acid	+58°	+82°	+86°	+86°

Cinchonidine (+)-α-(2-thienyl)hydrocinnamate crystallises as colourless needles melting at 137°.

2.8 g (0.012 moles) of benzylthienylacetic acid ($[\alpha]_D^{25} = -58°$), isolated from the first mother liquor above, and 2.6 g of *l*-ephedrine dihydrate were dissolved in a hot mixture of 43 ml of ethanol and 129 ml of water. The mixture was allowed to stand for 24 hours and the precipitate recrystallised until the optical activity of the liberated acid remained constant.

Crystallisation No.	1	2	3	4	5	6
ml of solvent (ethanol-water 1 : 3) . .	172	128	112	72	60	52
g of salt obtained	4.3	3.8	2.6	2.2	1.9	1.7
$[\alpha]_D^{25}$ of acid	−70°	−77°	−83°	−85°	−87°	−87°

l-Ephedrine (−)-*α-(2-thienyl)hydrocinnamate* crystallises as colourless thread-like crystal aggregates melting at 149°.

Resolution of 2-thenylphenylacetic acid: 6.9 g (0.03 moles) of optically inactive 2-thenylphenylacetic acid and 4.1 g (0.03 moles) of (+)-benzedrine were dissolved in a hot mixture of 125 ml of ethanol and 125 ml of water. The mixture was allowed to stand for 24 hours and the precipitate then filtered off and dried. The salt was recrystallised from dilute ethanol until the optical activity of the liberated acid remained constant.

Crystallisation No.	1	2	3	4	5
ml of solvent (ethanol-water 1 : 1) . .	250	136	96	76	64
g of salt obtained	5.9	4.2	3.4	2.8	2.4
$[\alpha]_D^{25}$ of acid (acetone)	+56°	+100°	+122°	+125°	+125°

(+)-*Benzedrine* (+)-*2-thenylphenylacetate* crystallises as long, colourless, shining needles melting at 184°.

3.5 g (0.015 moles) of thenylphenylacetic acid ($[\alpha]_D^{25} = -65°$) and 1.8 g (0.015 moles) of (+)-α-phenylethylamine were dissolved in a hot mixture of 55 ml of ethanol and 20 ml of water. The mixture was allowed to stand for 24 hours. The crystals were filtered off, dried and recrystallised from dilute ethanol until the optical activity of the liberated acid remained constant.

Crystallisation No.	1	2	3	4
ml of solvent (ethanol-water 5.5 : 2) . .	75	60	49	40
g of salt obtained	4.2	3.5	2.9	2.4
$[\alpha]_D^{25}$ of acid (acetone)	−112°	−122°	−128°	−127°

(+)-*α-Phenylethylamine* (−)-*2-thenylphenylacetate* crystallises as colourless shining needles melting at 185°–187°.

(+)-*α-(2-Thienyl)hydrocinnamic acid:* 4.8 g of the cinchonidine salt were decomposed with 2-*N* sulphuric acid and the liberated acid extracted with ether. After evaporation of the ether 2.0 g of crude acid remained. The acid was recrystallised several times from petrol (b.p. 60°–70°) and finally 1.6 g of pure acid was obtained as colourless needles. M.p. 71°–72°.

61.30 mg of acid, 5.88 ml of 0.04480–C NaOH.
$C_{13}H_{12}O_2S$. Equiv. wt. calc. 232.1, found 232.8.

97.3 mg in 10.01 ml of *acetone:* $2\,\alpha_D^{25} = +1.717°$, $[\alpha]_D^{25} = +88.3°$, $[M]_D^{25} = +205.0°$.
118.1 mg in 10.01 ml of *ethanol:* $2\,\alpha_D^{25} = +1.842°$, $[\alpha]_D^{25} = +78.1°$, $[M]_D^{25} = +181.2°$.
113.0 mg in 10.01 ml of glacial *acetic acid:* $2\,\alpha_D^{25} = +1.796°$, $[\alpha]_D^{25} = +79.6°$, $[M]_D^{25} = +184.6°$.
104.5 mg in 10.01 ml of *benzene:* $2\,\alpha_D^{25} = +1.567°$, $[\alpha]_D^{25} = +75.1°$, $[M]_D^{25} = +174.2°$.
114.3 mg of acid, dissolved in 2.0 ml of ethanol, were neutralised with 0.1–C NaOH and made up to 10.01 ml with *water:* $2\,\alpha_D^{25} = +1.531°$, $[\alpha]_D^{25} = +67.1°$, $[M]_D^{25} = +155.6°$.

(−)-α-*(2-Thienyl)hydrocinnamic acid:* 1.7 g of the *l*-ephedrine salt were decomposed with 2-*N* sulphuric acid and the liberated acid extracted with ether. From the ether layer 0.7 g of crude acid was obtained. After recrystallisation from petrol 0.5 g of acid remained. It resembles its optical antipode in all respects. M.p. 71°–72°.

51.09 mg of acid, 4.91 ml of 0.04480–C NaOH.
$C_{13}H_{12}O_2S$. Equiv. wt. calc. 232.1, found 232.5.

110.8 mg in 10.01 ml of *acetone:* $2\,\alpha_D^{25} = -1.942°$, $[\alpha]_D^{25} = -87.7°$, $[M]_D^{25} = -203.5°$.

(+)-*2-Thenylphenylacetic acid:* 2.4 g of the (+)-benzedrine salt were decomposed with 2-*N* sulphuric acid and the liberated acid was extracted and purified as described above. 1.0 g of pure (+)-acid were obtained as colourless needles, melting at 83°–85°.

40.14 mg of acid, 3.85 ml of 0.04480–C NaOH.
$C_{13}H_{12}O_2S$. Equiv. wt. calc. 232.1, found 233.0.

107.0 mg in 10.01 ml of *acetone:* $2\,\alpha_D^{25} = +2.714°$, $[\alpha]_D^{25} = +126.9°$, $[M]_D^{25} = +294.5°$.

(−)-*2-Thenylphenylacetic acid:* 2.4 g of the (+)-α-phenylethylamine salt were decomposed with 2-*N* sulphuric acid and the liberated acid extracted and purified as described for its optical antipode, which it resembles in all respects. M.p. 83.5°–85°.

53.40 mg of acid, 5.13 ml of 0.04480–C NaOH.
$C_{13}H_{12}O_2S$. Equiv. wt. calc. 232.1, found 232.6.

100.4 mg in 10.01 ml of *acetone:* $2\,\alpha_D^{25} = -2.565°$, $[\alpha]_D^{25} = -127.9°$, $[M]_D^{25} = -296.8°$.
96.5 mg in 10.01 ml of *ethanol:* $2\,\alpha_D^{25} = -2.198°$, $[\alpha]_D^{25} = -114.0°$, $[M]_D^{25} = -264.6°$.
98.0 mg in 10.01 ml of *benzene:* $2\,\alpha_D^{25} = -2.416°$, $[\alpha]_D^{25} = -123.4°$, $[M]_D^{25} = -286.4°$.
88.5 mg in 10.01 ml of glacial *acetic acid:* $2\,\alpha_D^{25} = -2.120°$, $[\alpha]_D^{25} = -119.9°$, $[M]_D^{25} = -278.3°$.
87.5 mg of acid, dissolved in 2.0 ml of ethanol, were neutralized with 0.1–C NaOH and made up to 10.01 ml with *water:* $2\,\alpha_D^{25} = -1.590°$, $[\alpha]_D^{25} = -91.0°$, $[M]_D^{25} = -211.2°$.

Melting-point diagrams

Thermal stability of the enanthiomorphs: Optically active acids were heated above their melting points (90°–100°) for about half an hour. The optical activities were measured in acetone before and after heating.

(+)-Thenylphenylacetic acid:

Before: 96.2 mg in 10.01 ml: $2\,\alpha_D^{25} = +2.36°$, $[\alpha]_D^{25} = +123°$.
After: 86.0 mg in 10.01 ml: $2\,\alpha_D^{25} = +2.08°$, $[\alpha]_D^{25} = +121°$.

(+)-Thienylhydrocinnamic acid:

Before: 101.8 mg in 10.01 ml: $2\,\alpha_D^{25} = +1.66°$, $[\alpha]_D^{25} = +81.5°$.
After: 89.9 mg in 10.01 ml: $2\,\alpha_D^{25} = +1.48°$, $[\alpha]_D^{25} = +82.3°$.

Melting-point preparations: Weighed amounts of the components (total weight 10 mg) were dissolved in a few drops of acetone ("AnalaR" standards for laboratory chemicals). The solvent was removed and the residue carefully powdered and dried in a desiccator. The melting points were determined in a hot-stage microscope (*4*).

Table 3. (+)- and (−)-Thienylhydrocinnamic acid.

% (−)-acid	M.p. °C
0.0	71 –72
10.0	64 –68.5
15.0	63.5–66.5
20.5	64 –65.5
25.0	63.5–68
30.5	63.5–70.5
35.5	63.5–72
44.0	64 –74
50.0	74 –75

Table 4. (+)- and (−)-Thenylphenylacetic acid.

% (+)-acid	M.p. °C
50.0	70.5–72
45.0	67.5–71.5
40.0	67 –71
34.5	67 –69.5
30.0	67 –70
25.0	67 –72.5
20.0	67 –75
10.0	67.5–80
0.0	83 –85

Table 5. (+)-Thienylhydrocinnamic acid (I) and (−)-thenylphenylacetic acid (II).

Mole-% (I)	M.p. °C
0.0	83–85
9.5	–80
19.5	–75
29.5	53–70
39.5	53–64
49.0	54–57.5
60.0	52–57
69.0	52–61
79.5	–65
87.0	53–67.5
100.0	70–72

Table 6. (+)-Thienylhydrocinnamic acid (I) and (+)-thenylphenylacetic acid (II).

Mole-% (I)	M.p. °C
0.0	83 –85
13.0	–80.5
21.0	–78
29.0	–75.5
31.5	63 –74.5
35.0	–73.5
37.5	–72.5
41.0	63 –71.5
51.0	63 –68
58.5	63 –65.5
69.5	63 –67.5
79.5	63 –69
89.5	63 –70.5
100.0	70.5–72

Summary

α-(2-Thienyl)hydrocinnamic acid and 2-thenylphenylacetic acid have been resolved into optical antipodes. The optically inactive acids are true racemates. Thermal analysis of the four possible two-component systems of the enanthiomorphs shows that the acids are unlikely to form a quasi-racemate. Measurements of the optical activities in different solvents indicate that the acids with the same direction of rotation belong to the same steric series.

The author wishes to express his thanks to Professor ARNE FREDGA for valuable discussions in connection with the present work. Part of the expenses has been defrayed by a grant from the Nobel Fund of the Royal Swedish Academy of Science (Kemiska prisgruppens särskilda fond).

Organic Department, Chemical Institute, University of Uppsala, January 1954.

REFERENCES

1. FREDGA, A., and PETTERSSON, K., Acta Chem. Scand. *4*, 1306 (1950).
2. PETTERSSON, K., Acta Chem. Scand. *4*, 395 (1950).
3. MATELL, M., Acta Chem. Scand. *7*, 698 (1953).
4. KOFLER, L. and A., Mikro-Methoden zur Kennzeichnung organischer Stoffe und Stoffgemische. Innsbruck 1948.
5. FREDGA, A., "The Svedberg 1884 $^{30}/_{8}$ 1944", Uppsala 1944, p. 261.
6. MATELL, M., Stereochemical studies on plant growth substances. Diss. Uppsala 1953, p. 23.
7. PETTERSSON, K. To be published.
8. FREDGA, A., Studien über Selen-di-karbonsäuren und Diselen-di-karbonsäuren. Diss. Uppsala 1935, p. 185.

Tryckt den 24 maj 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 33

Communicated 24 February 1954 by KARL MYRBÄCK

Note on the kinetics of exchange of isotopes with appreciable isotope effect

By LARS MELANDER

It is well known that the exchange of isotopic atoms or other species between two substances follows first-order kinetics, provided no ordinary chemical reactions disturb the system, there is no isotope effect, and the exchanging atoms are chemically equivalent within either substance. If the last condition is interpreted as involving uniform isotopic distribution within each phase at any instant, the proposition holds true for exchange in a heterogeneous system as well as in a homogeneous one. This relation is found in several papers (1).

HARRIS (2) has treated the case when appreciable isotope effects are involved, and concluded that the exchange kinetics approximate the first order even in this case, if the relative concentration of the distinguishable isotope is small enough. Since the approximation is justified in almost all work with radioactive tracers, for instance H^3 and C^{14}, the relation is very important. Harris based his calculations on a knowledge of the reaction mechanism. It is of interest, however, to see what conclusions can be drawn without such a knowledge, since isotopic exchange reactions are frequently studied in order to investigate the mechanism of the primary exchange.

In a particular system two kinds of molecules, A and B, are assumed to be present in constant macroscopic amounts, either in the same homogeneous phase or in two different phases with perfect mixing within each phase. Some atom or other entity will pass from A, where it is present in a molar amount a, to B, where it is present in a molar amount b, with a constant rate, measured in molar amount per unit of time, and simultaneously a corresponding amount will of course be transferred in the opposite direction, from B to A. The rate is assumed to be R for the unobservable exchange of ordinary entities. All exchangeable species should occupy equivalent positions within each kind of molecule.

The very small fractions of tracer atoms in A and B are denoted by y and z, respectively, and at the time origin we have for simplicity $y = y_0$ and $z = 0$.

The isotope effect for the transfer $A \to B$ is denoted by α, and that for the transfer $B \to A$ by β. The transfer rate in molar amounts of the distinguishable species will then be $y\alpha R$ and $z\beta R$, respectively.

In calculations of this kind it is generally assumed that the amount of material present in reaction intermediates is negligible, an assumption certainly valid in most cases. Then we have

$$y a + z b = y_0 a$$

and

$$-\frac{d(y a)}{d t} = y \alpha R - z \beta R$$

or

$$\frac{d y}{d t} = \frac{R}{a b}[\beta a y_0 - (\beta a + \alpha b) y].$$

Integration of this expression gives

$$\frac{y}{y_0} = \frac{\beta a + \alpha b\, e^{-\frac{\beta a + \alpha b}{a b} R t}}{\beta a + \alpha b}.$$

It follows that for infinite time $z_\infty / y_\infty = \alpha/\beta$, this constant determining the isotope distribution at equilibrium.

The fractional exchange, $(y_0 - y)/(y_0 - y_\infty) = F$, is sometimes a useful quantity, and the following simple relation holds:

$$-\ln(1 - F) = \frac{\beta a + \alpha b}{a b} R t.$$

Owing to the occurrence of reactions other than exchange in real systems it might sometimes be impossible to wait for complete exchange equilibrium to be attained. By observations during a much shorter interval, however, it might still be possible to find the initial decay rate of y and growth rate of z. The following relations hold:

$$\left[\frac{d}{d t}\left(\frac{y}{y_0}\right)\right]_{t=0} = \left[\frac{d}{d t}\left(\ln \frac{y}{y_0}\right)\right]_{t=0} = \left[\frac{d}{d t}(\ln y)\right]_{t=0} = -\frac{\alpha R}{a}$$

$$\left[\frac{d}{d t}\left(\frac{z}{y_0}\right)\right]_{t=0} = +\frac{\alpha R}{b}.$$

Thus these measurements give αR, and if similar measurements are carried out with the distinguishable isotope initially present in B, βR is found. The ratio will give the exchange equilibrium constant α/β. The latter could be used for the calculation of y_∞.

The knowledge of αR or βR is useful for the investigation of the exchange reaction mechanism. In general R is constant only for constant amounts a and b, other experimental conditions also being unchanged. The functional dependence of R on concentrations etc. could be found by variation of these independent quantities, provided the isotope effects are unchanged, which should be true if a single mechanism is operative. On the other hand the absolute magnitude of R, which is the exchange rate of the main isotope, cannot be determined by experiments with a single distinguishable isotope. The same is true of the isotope effects on rate, α and β, the determination of which amounts to the same problem. When two tracer isotopes are available, as in the case of hydrogen and carbon, it is possible to obtain an idea of the influence of mass on α and β, since R is the rate of exchange of the main isotope, irrespective of which tracer is used.

Nobel Institute of Chemistry, Stockholm 50, Sweden.

REFERENCES

1. For pertinent references, see A. A. Frost and R. G. Pearson, Kinetics and Mechanism, New York (1953), pp. 178–9.
2. G. M. Harris, Trans. Faraday Soc. *47* (1951), 716.

Tryckt den 24 mai 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 34

Read 10 February 1954

Properties of Lintner starches

By Karl Myrbäck and Ebba Willstaedt

With 9 figures in the text

A simple method for the preparation of "soluble starch" was indicated by C. J. Lintner in 1886 (1): Native starch is mixed with a sufficient volume of diluted hydrochloric acid (N acid according to the original prescription) and left for about two weeks at room temperature. The starch is then filtered off, washed with water until free from acid and dried in air at ordinary or only very slightly elevated temperature. On superficial inspection the product does not seem to differ materially from the original native starch. Microscopically the starch granules appear fairly unaltered as to size and shape. They are quite insoluble in cold water but swell and dissolve on boiling to more or less clear solutions of relatively low viscosity. If the acid treatment has been powerful enough no retrogradation of the solutions takes place on standing, at least no retrogradation visible to the eye. Granules and solutions of ordinary Lintner starches are coloured blue by iodine as with native starch. For general information on starches and conversion products, cf. (2).

Obviously the tranformation of the native, paste-forming starch into soluble Lintner starch is an acid hydrolysis of the native giant molecules into smaller fragments ("dextrins") yielding solutions of lower viscosity. However, the average degree of polymerization (DP) of an ordinary Lintner starch must still be fairly high since the product has a very low reducing power. In the diluted acid that can be used for the preparation of Lintner starch, no reactions other than hydrolysis of certain glucosidic linkages can be thought to occur. For this reason the Lintner starch, which may be characterized to a certain extent by its DP or reducing power, respectively, would, for most purposes, be preferred to preparations such as Zulkowski starch, containing glucosidically bound glycerol (Berner, 3), or "soluble starches" prepared by dry heating of native starch, in which loss of water through anhydride formation may occur.

Our interest in the Lintner starch originated from experiments reported in a series of papers from this laboratory (4) on the kinetics of malt α-amylase action on starches and their components and degradation products. It was concluded that the peculiar form of the hydrolysis curve observed with this enzyme and with native starch, amylose, glycogen, or amylopectin β-dextrin as substrates (4,5), could be ascribed to the mode of attachment of enzyme and substrate and to the different degrees of ramification of the substrates (6). However, in several papers by K.H. Meyer and his associates (e.g. 7) the correctness of our explanation was questioned and the form of our hydrolysis curves, and especially the sudden slowing off of the hydrolysis rate at a certain degree of hydrolysis, was thought to be caused by retrogradation of the substrates which admittedly renders them less susceptible to the amylase action. Several reasons, however, speak strongly against the MEYER theory, e.g. the fact that the hydrolysis rate of amylose slows down at a higher degree of hydrolysis than that of starch or glycogen (5), whilst it is a well-established fact that amylose solutions retrograde very much more rapidly than do solutions of the other substrates. Nevertheless, it seemed worth while to compare the kinetics of the enzyme action on starch with that of the action on soluble starches of different DP, even in cases where a retrogradation can not possibly play any role. Therefore, a number of Lintner starches were prepared and the hydrolysis by malt α-amylase was studied.

Disregarding the problem of the amylase action, there are also a number of other problems concerning the Lintner starch which should be of interest in studying the question of starch structure and the nature of the native starch granules. For instance, it can easily be shown that in the preparation of Lintner starch a portion of the granule substance by and by goes into solution. Nothing seems to be known about the nature of the dissolved substances. It could be asked, for instance, whether the dissolution consists in liberation of glucose from the granule surface or in the setting free of larger dextrin molecules. Furthermore, since the Lintner starch, remaining in solid form after intensive acid treatment and washing, is soluble in hot water and has a measurable and sometimes not at all small reducing power (see below), hydrolysis of certain linkages of the molecules must obviously take place *in situ*, and the forces holding the chain molecules together in the native granule must be capable of also holding the comparatively short-chain hydrolysis products together in such a way as to cause insolubility of the structure in cold water. Now it is clear that an increase in the time of acid treatment or an increase of the acid concentration must eventually lead to a total solubilization of the granules. However, it is not clear if this is brought about by the continuous liberation of small soluble molecules until the whole granule is eaten up, or by the simultaneous hydrolysis of linkages in the chain molecules of the solid structure. This hydrolysis must gradually weaken the forces holding the structure together, and it may well be that when a certain minimum average chain-length has been reached, the whole structure collapses, causing the remaining comparatively large portion of the granule suddenly to go into solution.

In this connection it should be mentioned that the hydrochloric acid used for Lintner starch preparation must not exceed *ca.* 12 per cent in concentration. If, for instance, a 15 per cent acid is used, the starch will, more or less rapidly, form a paste that cannot be filtered. In 25 per cent acid a thick paste is formed instantaneously, obviously because the hydrogen bonds keeping the polysaccharide chains together in the granule, are weakened at high ionic strength of the surrounding solution. Concerning granule structure, swelling etc., cf. (2).

It is very difficult, not to say impossible, to remove all traces of chloride ion from Lintner starch. In some cases, as for instance in work on the chloride-dependent animal amylases, it may be necessary to use starch or soluble starch free from chloride. In these cases, starch of the Lintner type may be prepared with *N* sulphuric instead of hydrochloric acid.

Experimental

Preparation of Lintner starch

Series 1: 200 g portions of native potato starch were mixed with 300 ml 2 per cent hydrochloric acid and stored in closed vessels at 20° C. The mixtures were stirred thoroughly twice a week. After a certain number of days a sample was filtered by suction. The filtrate was set aside for determination of sugar content etc., and the remaining starch was washed with destilled water until no chloride ion could be demonstrated in the washwater. The starch was then dried in air at room temperature. Dry weight was determined after drying for 20 hours at 100°.

Series 2: Same as series 1, but 10 per cent hydrochloric acid.

Investigation of filtrates

Reducing power was determined according to the Bertrand or Somogyi methods. All values are calculated as glucose.

Total amount of carbohydrate was determined by estimation of glucose after complete hydrolysis: N acid, 100°, 5 hours.

Average degree of polymerization (DP) was calculated from the reduction values before and after hydrolysis.

Optical rotation of the filtrates was determined in 1 dm tubes and sodium light, the specific rotation was calculated from $[\alpha]_{\mathrm{D}}^{20^\circ} = \alpha\ c$, where α is the registered angle and c = mg glucose/ml after complete hydrolysis.

Precipitation of saccharides from the filtrates. A portion of the filtrate was mixed with 5 volumes 99.5 per cent ethanol and left at +3° for about 20 hours. The precipitate was centrifuged off, washed with ethanol and ether and dried *in vacuo*.

Investigation of Lintner starch

Microscopical examination. Dry specimens and specimens soaked in water for a few minutes were examined and compared with samples of the original potato starch. Samples were also investigated after soaking in 0.01 N iodine-KJ solution.

Viscosity. A 2 per cent solution was prepared by heating a stirred suspension of starch in water for 45 minutes in a boiling water bath. The viscosity was determined immediately after cooling in a Hoeppler viscometer at 20°.

Reducing power was determined according to the Somogyi method on solutions freshly prepared by boiling.

Hydrolysis by amylases. A suitable amount of starch, Lintner starch or precipitate from the acid extracts was dissolved by boiling and brought to 90 ml volume. Buffer (phosphate or acetate) was added and the mixture was temperated in a water thermostate at 30°. The reaction was started by addition of the enzyme solution: total volume 100 ml. The increase of the reducing power was determined, generally according to the Willstätter and Schudel method. It was calculated as glucose in per cent of the theoretically possible yield. The values represent the number of ruptured linkages in per cent of the total number of glucosidic linkages in the substrate.

Barley β-amylase. Partly purified preparation from ungerminated six row barley or in some cases from soya beans. Traces of α-amylase had been removed in the conventional way by storing the solutions at pH = 3.6.

Malt α-amylase. Partly purified preparation from barley malt. β-Amylase had been removed by heating for 15 minutes at 70°. Both amylases were stored as dry powders and solutions of suitable strength were made up when needed.

Determination of glucose and maltose. These determinations were carried out in a few cases after amylase treatment of starch samples. The method (fermentation by baker's yeast) has been described previously (8).

Iodine reaction. Solutions containing 1 mg dry starch/ml were prepared by boiling and used immediately after cooling for determination of the iodine reaction. The iodine solution contained 0.200 g iodine in 100 ml 2 per cent potassium iodide solution.

A certain amount of the starch solutions, generally 15 ml, was introduced in a 500 ml volumetric flask and diluted with water to about 490 ml. Iodine solution, generally 5 ml, was added and the volume was adjusted to 500 ml. The blue, violet or reddish colour developed immediately and was stable for hours. The optical densities D at different wavelengths was determined in 1 cm cuvettes in a Beckman spectrophotometer, Model DU.

Results

I. Native potato starch

The potato starch used throughout this investigation was normal in appearance and had no measurable reducing power. The microscopic picture was normal: the size of the granules varied over a wide range (*ca.* 10–100 μ). The concentric striations and the *hilum* were very clearly visible on almost every granule.

The *viscosity* of a 2 per cent paste, prepared by boiling for 5 minutes was 52.5 cp. This value depends, of course, strongly on the time and mode of heating, stirring etc.

Colour with iodine. A solution of 15 mg starch in 495 ml water was prepared by boiling. Iodine solution was added and the volume was adjusted to 500 ml. Optical density was determined; density curves in Fig. 1. Absorption maximum at about $\lambda = 620$ mμ.

The absorption curve of the iodine solution alone is curve 1 in Fig. 1. In the range $\lambda = 300$ to about 375 mμ the absorption curves of the blue starch-iodine solutions coincide with the absorption curve of the iodine solution alone: Table 1. The explanation may be that only a very small portion of the added iodine is really bound by the starch. It seems possible, therefore, at least in experiments with high iodine concentrations, to correct the measured density values of the starch-iodine solutions by subtracting the corresponding values of the iodine solution. This has been done in Table 1 and in Figs. 1, 3 and 8. For $\lambda > 575$ mμ no correction is necessary.

Hydrolysis by malt α-amylase

Experiment 1: A paste was prepared by boiling in the usual way. 90 ml (dry weight 2.26 per cent) was mixed with 5 ml 0.3 M phosphate buffer, pH = 5.3. At 30° 5 ml enzyme solution was added and the increase of the reducing power was determined. Table 2 and Fig. 2 (○). *Expt.* 2: Duplicate of expt. 1. Table 2 and Fig. 2 (●). *Expt.* 3: Same as expt. 1, but 5 ml 2 M acetate buffer, pH = 5.3. Table 2 and Fig. 2 (+). *Expt.* 4: 11 g starch was suspended in 420 ml water and 40 ml 2 N sodium hydroxide was added with vigorous stirring. The paste was stirred for further 15 minutes and was then acidified to pH = 5 by the addition of 40 ml 3.6 N acetic acid. 95 ml was mixed immediately with 5 ml enzyme solution at 30°. Table 2 and Fig. 2 (□). *Expt.* 5: The same alkali treatment as in expt. 4, but the paste was acidified after 24 hours at 20°. Table 2 and Fig. 2 (△). *Expt.* 6: Wheat starch, otherwise as in expt. 1. Table 2 and Fig. 2 (×).

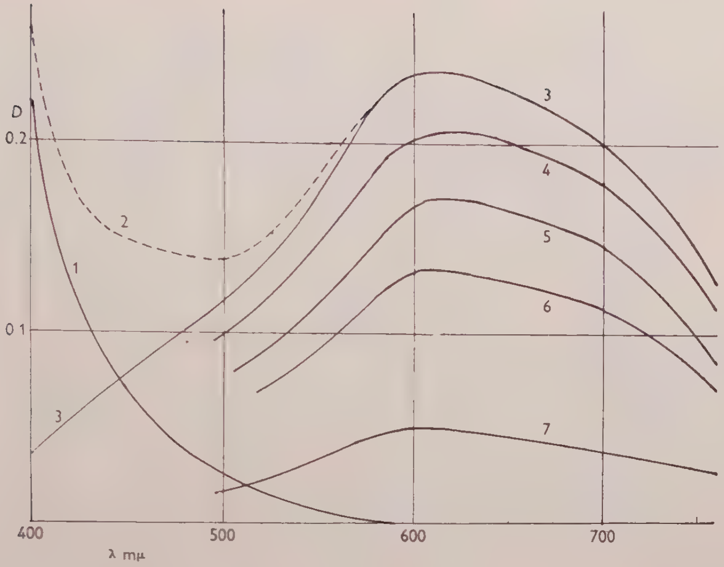


Fig. 1. Native starch and iodine. Optical density D at different wavelengths.
Curve 1: 4.6 ml iodine solution in 500 ml.
2: 4.6 ml iodine + 15 mg starch in 500 ml.
3: The same corrected for D of iodine solution.
4: 15 mg starch + 1.6 ml iodine in 500 ml (corr.).
5: 15 ,, ,, + 0.6 ,, ,, ,, ,, ,, ,,
6: 15 ,, ,, + 0.3 ,, ,, ,, ,, ,, ,,
7: 15 ,, ,, + 0.1 ,, ,, ,, ,, ,, ,,

Table 1. Optical density at different wave lengths of iodine and starch-iodine solutions.

Wave length mμ	Iodine solution 4.6 ml in 500 ml	Starch + iodine 15 mg + 4.6 ml in 500 ml	
		Measured	Corrected
335	0.690	0.705	0
350	0.885	0.885	0
365	0.759	0.759	0
375	0.593	0.593	0
380	0.474	0.490	0.016
400	0.219	0.258	0.039
425	0.110	0.168	0.058
450	0.071	0.149	0.078
475	0.043	0.143	0.100
500	0.024	0.138	0.114
525	0.014	0.152	0.138
550	0.007	0,182	0.175
575	0.002	0.218	0.216
600	0	0.236	0.236
625	0	0.237	0.237
650	0	0.228	0.228
700	0	0.200	0.200
750	0	0.142	0.142
800	0	0.070	0.070

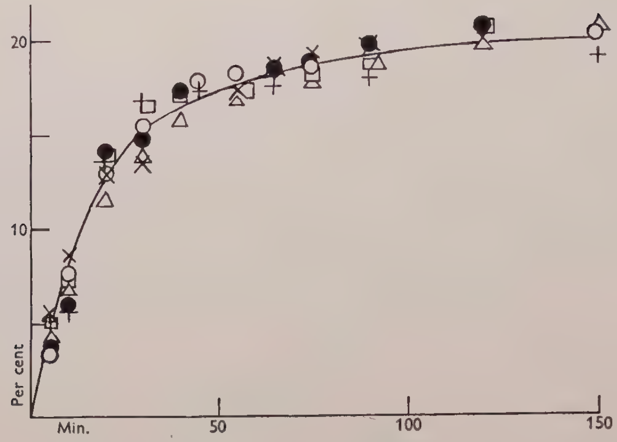


Fig. 2. Hydrolysis of native potato starch by malt α-amylase.

Table 2. Hydrolysis of native starch by malt α-amylase.

Reaction time	Hydrolysis in per cent of theoretical					
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6
30 sec.	1.8	2.5	—	2.4	2.2	—
5 min.	3.4	3.9	5.1	5.1	4.2	5.6
10 ,,	7.8	5.9	5.7	7.1	6.8	8.7
20 ,,	13.0	14.3	13.6	13.7	11.5	13.0
30 ,,	15.6	14.8	16.8	16.5	13.7	13.4
40 ,,	—	17.3	—	17.1	15.6	—
45 ,,	17.8	—	17.2	—	—	—
55 ,,	18.2	—	—	17.6	16.6	17.5
65 ,,	—	18.5	17.4	—	—	18.7
75 ,,	18.4	18.8	—	18.1	17.7	19.2
90 ,,	—	19.7	17.8	18.5	18.6	19.5
120 ,,	—	20.5	—	20.4	19.5	19.8
150 ,,	20.0	—	18.8	—	20.4	—
210 ,,	—	20.7	19.0	—	20.8	—
240 ,,	—	—	—	—	—	22.8
20 hrs.	27.4	—	24.4	—	25.0	—
23 ,,	—	—	—	—	—	32.0

Fig. 2 shows that the hydrolysis curve is practically the same irrespective of buffer and pretreatment of the substrate solution. The experiments are quite in accordance with earlier results from this laboratory, and they support the view that the kinetics of the malt amylase action depends chiefly on the chemical structure of the substrate rather than on its physical condition.

II. Lintner starches prepared in 2 per cent hydrochloric acid

A few samples were prepared in 2 per cent HCl. Some properties of the starches and the filtrates are recorded in Table 3. The values of the reducing power have been determined by the Somogyi method; the values found for starches Nos. I:1–I:4 have been excluded since it is known that the presence of large amounts of starch causes considerable errors when the reducing power is very low.

Table 3. Properties of Lintner starches and filtrates.

Series I.

Starch No.	Acid treatment days	Reducing power, per cent "glucose"	Viscosity, 2 % solution, cp	$[\alpha]_D^{20^\circ}$	Iodine reaction	
					D_{max}	λ_{max} mμ
Native starch	0	0	52.5	—	0.238	620
I: 1	1	~0	18.7	—	0.276	605
I: 2	2	~0	11.8	—	—	—
I: 3	4	~0	13.0	—	—	—
I: 4	5	~0	5.6	—	0.267	595
I: 5	12	~0.5	2.9	—	—	—
I: 6	32	0.89	1.36	—	0.248	585
I: 7	129	1.98	1.19	—	0.225	565
I: 8	261	2.42	1.31	—	0.192	565
I: 9	271	2.07	1.31	~195°	0.192	555
I: 10	431	3.19	—	—	—	—
I: 11	440	2.75	1.50	195°	0.187	550

Filtrates.

From Starch No.	Yield, per cent	Reducing power, per cent "glucose"	Average chain length, $\bar{n}$	$[\alpha]_D^{20^\circ}$
I: 7	—	21.5	4.7	166°
I: 8	—	20.2	5	—
I: 9	—	25.2	4	172°
I: 10	11.9	—	—	—
I: 11	12	26.4	3.8	145[1]

[1] Fermentation experiments showed that about 25 per cent of the dissolved substance was glucose. Other fermentable sugars were present only in very small amounts.

The viscosity decreases rapidly. A definite end-value of about 1.3 cp seems to be reached (compare series 2, Table 15).

As could be expected, the values of the specific rotation of starch do not undergo any noticeable change during the acid treatment. The rotation of the filtrates, on the other hand, is considerably lower, corresponding to the low average chain lengths. The filtrates contain only moderate amounts of glucose and generally small amounts of other fermentable sugars. The non-fermentable dextrins have strongly varying chain lengths. Fractions of considerably higher average DP can be isolated, for instance by alcohol precipitation (see below). Since hydrolysis of the dissolved saccharides obviously continues in the acid solution, we may conclude that from the granules (which superficially do not change their appearance very much) not only glucose and oligosaccharides but also rather long chain molecules, sometimes containing at least 20 glucose units, are leached out by the acid.

Microscopical examination

Lintner starch preparations I:1–I:6 have a rather normal appearance. However, the fact that the striations, clearly visible on the surface of the native granules, have completely vanished, shows that a dissolution of the surface layers has taken place. It may even be that some of the smallest granules have dissolved completely. From preparation I:7 onwards the granules appear more and more disfigured. Deep fissures appear, eventually causing the granules to split in halves and so on. In the granules of the two last preparations of Table 3 the fissures are many and deep, and most granules are split up in several small pieces having a rather "amorphous" appearance. They are coloured more or less reddish by dilute iodine solution.

Binding of iodine by the granules

A few experiments were carried out to determine the binding of iodine to the granules. One gram samples of the various starches were gently shaken with 100 ml 0.0076 *N* iodine solution, containing 2 g KJ/liter. After a certain time the suspensions were filtered and iodine was determined in the filtrates. Two experiments in Table 4. The amounts of bound iodine are very small, and the binding is probably to be classified as a surface adsorption. Therefore, it does not seem very probable that determination of the binding capacity of the granules for iodine could contribute very much to the characterization of the various starches. As seen from Table 4, the difference between the values for native starch and for a strongly degraded Lintner starch are measurable but rather small.

Table 4. Binding of iodine to starch granules.

Native starch		Lintner starch I: 9	
Time of treatment	Iodine bound, mg/g	Time of treatment	Iodine bound, mg/g
1–2 min.	2.86	1–2 min.	2.32
5–6 ,,	3.52	30 ,,	2.60
20 ,,	3.60	2 hrs.	2.98
30 ,,	3.80	18 ,,	4.00
70 ,,	4.00		
16 hrs.	4.32		

Iodine reaction in solution

Solutions containing 1 mg (dry weight) starch/ml were prepared. 15 ml was diluted in a 500 ml volumetric flask to 495 ml and 5 ml iodine solution was added. The density curves (corrected as above for the absorption of the iodine solution) for the Lintner starches of series 1 are collected in Fig. 3. The density (colour intensity) decreases

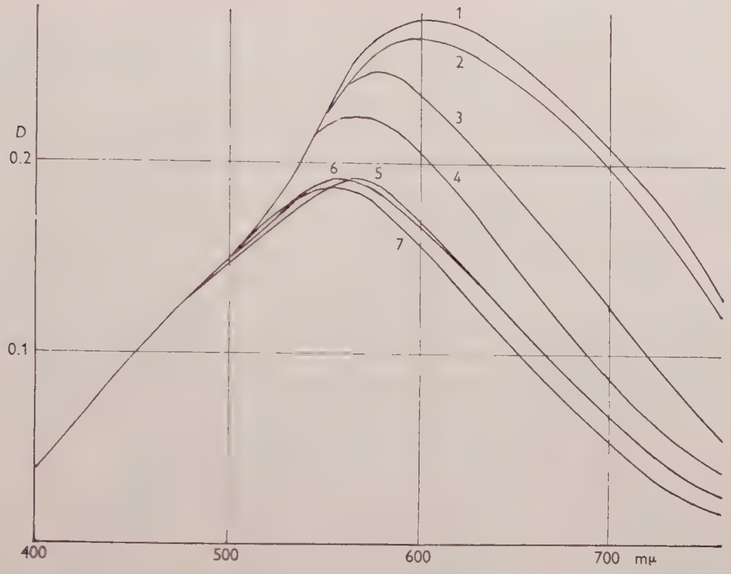


Fig. 3. Lintner starches and iodine. Optical density at different wavelengths.

Curve 1: Lintner starch I: 1		Curve 5: Lintner starch I: 8
2: ,, ,, I: 4		6: ,, ,, I: 9
3: ,, ,, I: 6		7: ,, ,, I: 10
4: ,, ,, I: 7		

regularly with decreasing DP of the starch. Simultaneously the position of the maximum is shifted from about 620 mμ for native starch to about 550 mμ.

Action of amylases on the Lintner starches

Lintner starch I: 3

Action of malt α-amylase. Expt. 1: 90 ml 2.5 per cent starch solution + 5 ml phosphate buffer, pH = 5.3 + 5 ml enzyme. Table 5. *Expt. 2*: Same as expt. 1, but 1.8 per cent starch and pH = 5.6. Table 5. *Expt. 3*: Same as expt. 2, but enzyme treated with 15 mg cysteine at pH = 5.5 for 10 minutes before addition of the substrate. Table 5. *Expt. 4*: 95 ml *ca.* 5 per cent starch solution + 5 ml amylase solution; Table 5. After 21 hours 80 ml of the mixture, corresponding to 3.66 g dry weight, was boiled to destroy the enzyme, and the volume was reduced to 25 ml. The solution was precipatated in fractions with acetone and ether. Thirteen fractions were obtained; each precipitate was collected by centrifugation, suspended in acetone, centrifuged again after 20 hours, suspended in ether, centrifuged, dried and dissolved in *ca.* 50 ml water. The solutions were boiled to remove all organic solvents and brought to 10 ml volume. Reducing power before and after complete hydrolysis was determined according to the WILLSTÄTTER and SCHUDEL method. Average DP and yields in Table 6.

The total yield of dextrin fractions was 72.4 per cent. The distribution on various chain lengths is shown in Fig. 4. The last three precipitates and the non-precipitable fraction obviously contain chiefly maltose together with small amounts of glucose and possibly maltotriose and isomaltose. The distribution of the dextrins on chain lengths of three or more glucose units is very similar to that found after dextrinization of native starch with malt α-amylase. In this case, if the en-

Table 5. Action of malt α-amylase on Lintner starch I: 3.

Experiment 1		Experiment 2		Experiment 3		Experiment 4	
Time	per cent glucose	Time	per cent glucose	Time	per cent glucose	Time	per cent glucose
2 min.	3.2	2 min.	3.0	2 min.	2.0	30 min.	4.1
4 ”	5.1	5 ”	5.2	5 ”	4.0	60 ”	9.6
6 ”	5.8	12 ”	8.4	10 ”	7.3	75 ”	12.3
10 ”	7.7	28 ”	13.2	24 ”	13.6	90 ”	12.8
14 ”	11.0	44 ”	14.8	40 ”	17.5	120 ”	13.0
22 ”	12.7	60 ”	16.2	60 ”	18.3	155 ”	14.0
35 ”	14.9	85 ”	18.1	95 ”	18.6	180 ”	15.0
40 ”	17.1	150 ”	20.1	19 hrs.	30.2	405 ”	19.5
55 ”	17.4	235 ”	23.1			21 hrs.	26.1
65 ”	17.7	24 hrs.	29.1				
90 ”	17.7						
120 ”	17.7						
180 ”	18.8						
18 hrs.	28.2						

Table 6. α-Dextrin fractions from Lintner starch I: 3.

Solvent added, ml	Reducing power (mg “glucose”)		DP	Yield in per cent of theoretical
	Before hydrolysis	After hydrolysis		
35 acetone	39.7	321	8.1	7.90
50 ”	33.3	316	9.4	7.78
60 ”	62.1	441	7.1	10.86
70 ”	41.4	250	6.1	6.16
85 ”	75.4	350	4.6	8.62
105 ”	59.5	246	4.1	6.06
125 ”	42.5	182	4.3	4.48
155 ”	43.3	131	3.0	3.23
205 ”	38.9	111	2.8	2.73
280 ”	35.3	106	2.9	2.61
30 ether	33.0	68	2.1	1.68
80 ”	51.0	112	2.2	2.76
205 ”	140	304	2.2	7.47
				72.35

zyme action is stopped after the initial rapid phase (dextrinization), i.e. at about 17 per cent theoretical glucose, it is found that the non-fermentable products, the α-dextrins, have chain lengths of about 6–10 units. If the enzyme action is allowed to proceed beyond the dextrinization stage a comparatively slow saccharification of the primary α-dextrins is observed. Dextrins with lower DP and fermentable sugars are formed in increasing amounts. Since the hydrolysis in expt. 4 (Table 5) had reached a value of 26 per cent, it is quite understandable that the amounts of sugars and low-molecular dextrins are fairly high.

Experiments 1–3 (Table 5) show that the hydrolysis of Lintner starch I:3 is represented by a curve identical with that of native starch. Since the viscosity of a 2

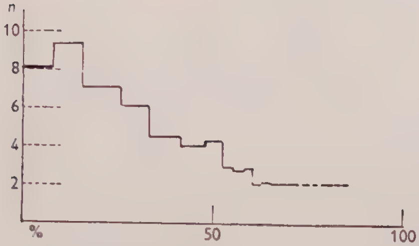


Fig. 4. Distribution of degradation products on different chain lengths (n).

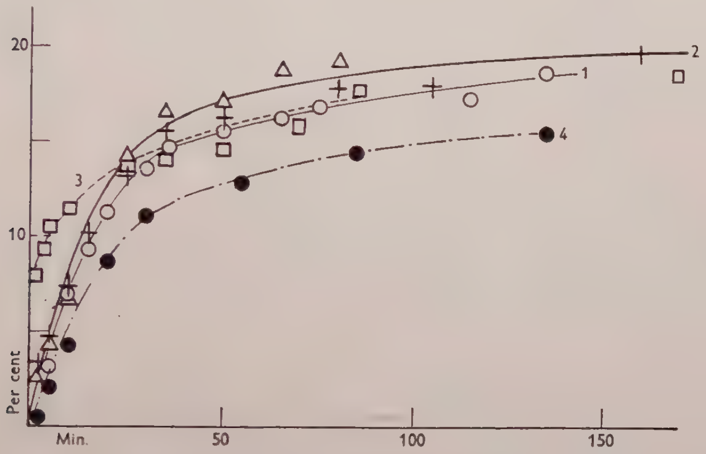


Fig. 5. Action of malt α-amylase.
Curve 1: Lintner starch I: 6.
2: Native starch.
3: Alkali-treated Lintner starch I: 6.
4: Lintner starch I: 8.

per cent solution of Lintner starch I:3 is only about one fourth of that of the corresponding solution of native starch, it does not seem possible that the peculiar form of the hydrolysis curve could be caused by the physical state of the substrate solution.

Lintner starch I: 6

Experiment 1: 90 ml 2.5 per cent starch solution + 5 ml phosphate buffer, pH = 5.3 + 5 ml malt α-amylase solution. Table 7 and curve 1 in Fig. 5 (○). The full-drawn curve 2 is that corresponding to native starch. *Expt. 2*: 85 ml 2 per cent starch solution + 10 ml 2 N acetate buffer, pH = 5.4 + 5 ml malt α-amylase solution. Table 7 and Fig. 5 (+). *Expt. 3*: 2 g Lintner starch was dissolved by boiling in 20 ml water. After cooling 5 ml 0.03 N sodium hydroxide was added and the mixture was kept at room temperature for 20 hours. It was diluted to 90 ml, temperated at 30° and added drop by drop to a stirred mixture of 5 ml 2 N acetate buffer (initial pH 4.6) and 5 ml malt α-amylase solution, kept in a thermostate at 30°. The addition took 10 minutes which are not included in the reaction times given in Table 7. Curve 3 in Fig. 5 (□).

It appears from Table 7 and Fig. 5 that the hydrolysis of Lintner starch I:6 fol-

Table 7. Action of malt α-amylase on Lintner starch I: 6.

Experiment 1		Experiment 2		Experiment 3	
Time	per cent glucose	Time	per cent glucose	Time	per cent glucose
5 min.	3.2	2 min.	3.3	2 min.	7.9
10 ,,	7.1	5 ,,	4.8	3 ,,	9.3
15 ,,	9.3	10 ,,	7.4	5 ,,	10.5
20 ,,	11.3	15 ,,	10.1	10 ,,	11.4
30 ,,	13.6	25 ,,	13.1	25 ,,	13.6
36 ,,	14.7	35 ,,	15.5	35 ,,	14.0
50 ,,	15.6	50 ,,	16.2	50 ,,	14.5
65 ,,	16.2	80 ,,	17.8	70 ,,	15.7
75 ,,	16.8	105 ,,	18.0	85 ,,	17.8
115 ,,	17.3	160 ,,	19.8	170 ,,	18.6
135 ,,	18.8	19 hrs.	28.8	260 ,,	20.2
21 hrs.	27.3			24 hrs.	28.1

lows practically the same curve as that of native starch. Even "rejuvenation" (7) of the substrate with sodium hydroxide and direct mixing of the rejuvenated substrate and the enzyme does not change the kinetics of the hydrolysis materially.

Lintner starch I: 7

Action of malt α-amylase on one per cent starch solution, phosphate buffer, pH = 5.4. Increase of reducing power (corrected for reducing power of starch) in Table 8 (2 experiments). The hydrolysis curve is the curve 1 in Fig. 6. Since this Lintner starch is fairly strongly degraded it appears to be excluded that retrogradation

Table 8. Action of amylases on Lintner starch I: 7 and on precipitate from acid solution.

Lintner starch + α-amylase			Precipitate from acid extract of starch				
Time	Increase of reducing power, per cent		Time	β-Amylase		α-Amylase	
	Expt. 1	Expt. 2		Reducing power, per cent	Increase of reducing power, %	Reducing power, per cent	Increase of reducing power, %
20 min.	9.0	9.8	0	9.2	0	9.2	0
40 ,,	14.5	14.6	5 min.	15.4	6.2	18.1	8.9
80 ,,	15.3	15.8	10 ,,	19.5	10.3	19.1	9.9
115 ,,	16.0	18.3	30 ,,	27.7	18.5	21.7	12.5
20 hrs.	27.3	30.3	2 hrs.	32.2	23.0	—	—
44 ,,	38.3	41.5	3 ,,	—	—	26.4	17.2
			21 ,,	38.7	29.5	40.0	30.8
			48 ,,	38.7	29.5	—	—
			120 ,,	41.5	32.3	50.8	41.6

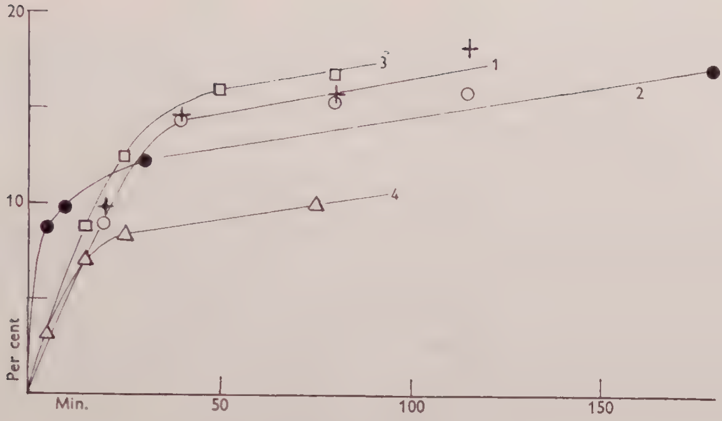


Fig. 6. Action of malt α-amylase.
Curve 1: Lintner starch I: 7.
2: Precipitate from acid solution, I: 7.
3: Lintner starch I: 11.
4: Precipitate from acid solution, I: 11.

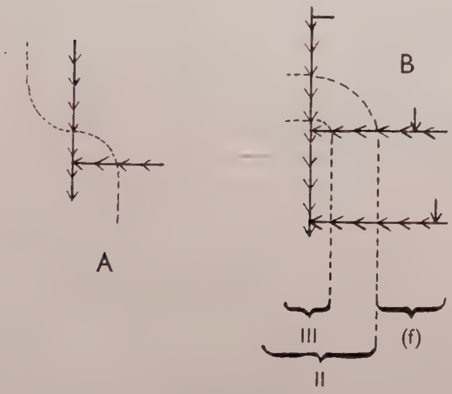


Fig. 7. Enzymatic degradation of certain dextrins.

could play any significant role in the course of hydrolysis. The hydrolysis curve has the same form as in the case of native starch and the various Lintner starches investigated in the foregoing.

Acid-soluble carbohydrate. The hydrochloric acid filtrate from Lintner starch I:7 contained dextrins with an average DP of about 4. A portion of the filtrate was precipitated with 5 volumes of ethanol; about 35 per cent of the dissolved substance was recovered. The reducing power of the precipitate (Somogyi method) corresponded to an average DP of about 11. The hydrolysis of this substance by β-amylase and malt α-amylase was investigated: Table 8.

The hydrolysis by β-amylase comprises approximately 30 per cent of the glucosidic linkages of the substrate and ceases at this value (= *ca.* 60 per cent maltose; no glucose was present). The fact that the same value is obtained with β-amylase and native starch shows that in the preparation precipitated from the acid extract, as in starch itself, *ca.* 60 per cent of the glucose residues are situated in the non-reducing end-chains of ramified molecules, or (less often) in non-ramified molecules. A probable structure of an average molecule of the preparation investigated is depicted in Fig. 7 *A*. The portion to the right of the dotted line will be split off as maltose by β-amylase (about 55 per cent).

The hydrolysis of the precipitated dextrin preparation by *malt α-amylase* is shown in Fig. 6, curve 2 (●). The curve has again the same general form as in the case of native starch and the more or less strongly degraded Lintner starches. The rapid dextrinization phase, however, is shorter and comprises some 10 per cent of the linkages. Since the average DP of the substrate was 11, this means that, on the average, one linkage per substrate molecule has been ruptured by the enzyme at a high rate while the hydrolysis of further linkages proceeds at a strongly reduced rate. This is quite in accordance with the theory of the malt α-amylase action put forward in several earlier papers from this laboratory.

Lintner starch I: 8

Action of malt α-amylase. 2 g Lintner starch in 90 ml, phosphate buffer, enzyme, total 100 ml. Table 9 and curve 4 in Fig. 5 (●). This curve has the same general form as that of native starch (curve 2), but the dextrinization phase comprises only about 13 per cent of the linkages as compared to about 17 per cent for native starch. As pointed out earlier, this must be expected as in the Lintner starch I:8 2.42 per cent of the linkages have already been ruptured by the acid treatment (Table 3).

Action of β-amylase. 85 g Lintner starch (dry weight) was dissolved in 1,700 ml water and treated with β-amylase until constant reducing power. Maltose was removed by baker's yeast. The filtrate was reduced to 300 ml and the remaining dextrin was precipitated with ethanol. 30 g (dry) β-dextrin, called β-dextrin I, was obtained, or about 35 per cent of the Lintner starch, i.e. about the same yield as that normally obtained from ordinary starch. The reducing power of β-dextrin I (Somogyi method) was 4.5 per cent apparent glucose. This is a reasonable value since the Lintner starch I:8 had a reducing power corresponding to 2.42 per cent glucose. Average DP of β-dextrin I = 24.

As expected, the β-dextrin I was completely stable against β-amylase. However, pretreatment of the dextrin with dried brewer's yeast made it again susceptible to β-amylase. The following series of experiments was carried out; cf. Fig. 7 B, in which a possible average structure of a β-dextrin molecule is depicted.

Table 9. Action of amylases on Lintner starch I: 8 and on β-dextrin.

Starch I: 8 and malt α-amylase				β-Dextrin II and β-amylase	
Time	per cent glucose	Time	per cent glucose	Time	g maltose
2 min.	0.6	85 min.	14.6	0	0.032
5 ,,	2.1	135 ,,	15.6	10 min.	0.069
10 ,,	4.3	210 ,,	17.1	30 ,,	0.087
20 ,,	8.8	305 ,,	22.6	2 hrs.	0.095
30 ,,	11.2	22 hrs.	23.8	20 ,,	0.097
55 ,,	12.9	46 ,,	28.5		

4.3 g β-dextrin I (dry weight) was dissolved in 85 ml water + 15 ml 10 per cent phosphate buffer, pH = 6.2 (9). 10 g dried brewer's yeast was added and the mixture was kept at 30° for 45 hours. The carbon dioxide evolution (which practically ceased after 20 hours) corresponded to fermentation of about 50 per cent of the β-dextrin I (*f* in Fig. 7 *B*). The mixture was filtered and washed; filtrate and washings were boiled for some minutes and filtered again. The volume was reduced to 100 ml. The reducing power corresponded to 0.168 g apparent glucose. Complete hydrolysis of a small sample showed the presence of 2.30 g "secondary β-dextrin" (β-dextrin II, Fig. 7 *B*). The average DP of this dextrin was thus about 14, compared to 24 in the primary β-dextrin I. Therefore, it seems that in the fermentation about 50 per cent of the glucose residues of every β-dextrin molecule has been released and fermented.

10 ml of the secondary β-dextrin solution (0.23 g) was brought to pH = 5.3, and 1 ml β-amylase solution was added. The increase of the reducing power was followed and calculated as maltose: Table 9. It appears that about 40 per cent of the β-dextrin II has been converted to maltose. The remaining "tertiary" β-dextrin III is stable to β-amylase because it has, like the primary β-dextrin I, ramified end-groups.

These experiments confirm those of MEYER *et al.* (10) which constituted one of the chief reasons for the assumption of the irregular and multiple ramification of the amylopectin molecules.

Since our primary β-dextrin I had an average DP = 24 and since the interior chains of the amylopectin appear to have an average chain length of about 6 glucose units, one of our β-dextrin I molecules may be schematically represented by the formula in Fig. 7 *B*. Since about 50 per cent was fermented by dried brewer's yeast, we have to conclude that 3–4 units of each non-reducing end-chain were removed in fermentation; the fermented portion is marked *f* in the figure. The DP of the secondary β-dextrin II should be 13. From this β-dextrin II β-amylase should be able to release 6 glucose units, or 46 per cent, as maltose, and the remaining β-dextrin III would contain 7 glucose units.

Table 10. Fermentation of maltose and β-dextrin.

	ml Carbon dioxide						
	10 min.	30 min.	1 h.	2 h.	3 h.	4 h.	20 h.
100 mg maltose hydrate	0.8	9.5	16.4	21.5	23.6	24.0	24.0
88 mg β-dextrin . . .	0.3	0.7	1.0	2.0	2.9	3.6	11.5

As pointed out by MEYER *et al.* (10) it must be assumed that the dried brewer's yeast is able to release terminal glucose residues, united by α-glucosidic 1,6-linkages to the chains. However, since in our experiments as much as *ca.* 50 per cent of the β-dextrin I was fermented, the dried yeast is evidently also able to release end-groups united by 1,4-linkages. It must be assumed, therefore, that in our experiment fermentation ceased, not because of the structure of the remaining β-dextrin II, but because of other circumstances such as the destruction of some component of the fermentation system. The fermentation of the β-dextrin by dried brewer's yeast proceeds rather slowly compared to that of maltose (Table 10), and the enzyme inactivation may well play a part in the result. The following experiment was performed to test this possibility:

80 mg β-dextrin I was mixed with 2 ml 2.5 per cent phosphate buffer, pH = 6.2, and 0.2 g dried brewer's yeast; 20 hours at 30°. Fermentation had ceased completely and 11.2 ml carbon dioxide had been evolved. The mixture was filtered and washed, filtrate and washings were reduced to 2 ml volume and 0.2 g dried yeast was added; 20 hours at 30°. Fermentation had ceased and 6.0 ml carbon dioxide had been evolved. The procedure was repeated a third time, but no carbon dioxide evolution was observed (all values corrected for the yeast autofermentation). Fermentation of 100 mg glucose (fructose, maltose hydrate) by the same yeast yielded 24 ml CO_2 on the average. The 17.2 ml CO_2 from β-dextrin I would correspond to about 72 mg glucose. Thus it appears that the cessation of fermentation of the β-dextrin (and similar substrates) does not necessarily mean that all fermentable carbohydrates are removed; on the other hand, it does not seem possible to ferment the β-dextrin I completely by dried brewer's yeast. Only 72 mg out of 88 (calculated as glucose) were fermented by repeated treatments of the dextrin with new portions of yeast until no fermentation could be observed.

Since fermentations by dried brewer's yeast depend strongly on phosphate concentration and, moreover, can be modified in a remarkable way by arsenate, a number of fermentation experiments on β-dextrin I were performed with varying phosphate and arsenate concentrations. The yield of CO_2 from 88 mg dextrin in 2 ml is seen in Table 11 (values corrected for autofermentation). The yield increases with phosphate concentration. The values found in the presence of arsenate are a little erratic; however, moderate concentrations of arsenate seem to increase the yield of CO_2 somewhat. This being so, the above experiment with a threefold fermentation was repeated in the presence of 0.05 *M* arsenate. The total yield of CO_2 varied somewhat; up to 19 ml CO_2 was obtained. This would correspond to a fermentation of 79 mg glucose. It seems that a small portion of the β-dextrin is non-fermentable or at least very slowly fermentable. It must be admitted, however, that the calculation of fermented sugar from the volume of carbon dioxide is not very accurate. Nor can the presence of non-fermentable carbohydrates in the filtrates after fermentation be ascertained with any considerable exactness, since the filtrates contain rather large amounts of reducing substances originating from the yeast. This is seen from the following experiment:

(*a*) 88 mg β-dextrin I + 0.5 ml 0.3 *M* phosphate, pH = 6.2 + 1 ml 0.1 *M* arsenate + 0.5 ml water + 0.2 g dried brewer's yeast. Fermentation was repeated three times as above.

(*b*) Same experiment without dextrin. Results in Table 12.

Table 11. Fermentation of β-dextrin at various concentrations of phosphate and arsenate.

ml 0.3 *M* phosphate	ml 0.1 *M* arsenate	ml CO_2
—	—	5.6
0.05	—	6.3
0.1	—	8.0
0.3	—	9.0
0.5	—	10.0
0.7	—	12.8
1.0	—	12.8
1.3	—	12.8
0.2	0.02	9.4
0.2	0.05	12.5
0.2	0.1	12.4
0.2	0.2	8.3
0.2	1.0	14.3
0.5	0.1	11.1
0.5	0.2	10.6
0.5	0.5	13.4
0.5	0.8	11.6
0.5	1.0	15.8
0.5	1.3	12.6

Table 12. Fermentation of β-dextrin.

	ml Carbon dioxide			Non-fermentable dextrin	
	1st	2nd	3rd	Reducing power	
	fermentation			before	after
				hydrolysis	
Yeast + β-dextrin	12.7	7.1	4.2	6.5	34
Yeast	2.1	2.6	2.4	2.6	12

The corrected value for CO_2 is 16.9 ml, corresponding to *ca.* 70 mg glucose. Since 88 mg β-dextrin is equal to about 97 mg glucose, this would mean that *ca.* 27 mg of the dextrin were unfermentable or very slowly fermentable. The corrected value of carbohydrate in the filtrate after the fermentation is 22 mg. Although this value is far from accurate, the result appears to be that a portion of the β-dextrin ($\sim$ 25 per cent) is not or very slowly fermented. The mean DP of the unfermented residue would be *ca.* 4 units (Table 12). It is not possible to decide, on the basis of these experiments, why the saccharides of this fraction are not fermented. If they are thought to contain only 1,4-α- and 1,6-α-glucosidic linkages, it might be that not all of the possible isomeric tetrasaccharides (cf. (11) Fig. 2) are fermentable. Or the explanation could be that in the non-fermentable fraction (and thus in the amylopectin) other linkages than 1,4-α- and 1,6-α-linkages are present in small amounts.

Lintner starch I: 9

2 g Lintner starch in 85 ml water + 10 ml 2 N acetate buffer, pH = 5.4 + 5 ml malt α-amylase solution: Table 13 and Fig. 5 (△). The hydrolysis curve has quite the normal appearance and coincides practically with that of native starch (curve 2).

Table 13. Action of malt α-amylase on Lintner starch I: 9.

Time	per cent glucose	Time	per cent glucose
2 min.	2.7	50 min.	17.1
5 ,,	4.4	65 ,,	18.9
10 ,,	7.0	80 ,,	19.3
25 ,,	14.1	320 ,,	25.9
35 ,,	16.7	23 hrs.	35.2

Lintner starch I: 11

This strongly degraded starch was somewhat sticky after filtration and washing with water. It was therefore washed with 50 per cent ethanol, then with strong alcohol, and dried *in vacuo*. The reducing power (BERTRAND method) was 2.75 per cent, corresponding to an average DP = 36.

Action of malt α-amylase on 1 per cent starch solution; increase in reducing power in Table 14. The hydrolysis curve is shown in Fig. 6, curve 3 (□). It has the normal form.

Acid-soluble carbohydrate. A fraction of the substance dissolved in the hydrochloric acid was isolated by alcohol precipitation as described above. Average DP of the precipitate was 10. The action of β-amylase and of malt α-amylase on a solution of this fraction is shown in Table 14. The β-amylase experiment shows that, in the dextrin fraction, 51 per cent of the glucose units are situated in the non-reducing end-chains of ramified molecules or in non-ramified molecules. The α-amylase experiment (curve 4 in Fig. 6) shows that some 7 per cent of the linkages, i.e. on an average one linkage per dextrin molecule, is ruptured by the enzyme at a high rate, which is in agreement with the theory quoted above.

Table 14. Action of amylases on Lintner starch I: 11 and on precipitate from acid solution.

Lintner starch I: 11		Precipitate from acid solution			
α-Amylase		β-Amylase		α-Amylase	
Reaction time	Hydrolysis, per cent	Reaction time	Hydrolysis, per cent	Reaction time	Hydrolysis, per cent
15 min.	8.8	5 min.	7.1	5 min.	3.1
25 ,,	12.5	25 ,,	22.4	15 ,,	7.1
50 ,,	16.0	50 ,,	25.0	25 ,,	8.3
80 ,,	16.9	170 ,,	25.5	75 ,,	10.2
4 hrs.	20.5	20 hrs.	25.5	240 ,,	12.9
21 ,,	27.9	72 ,,	25.5	20 hrs.	19.3
220 ,,	52.4			45 ,,	25.5
				93 ,,	28.5

III. Lintner starches prepared in 10 per cent hydrochloric acid

Some properties of these starches and of the filtrates are recorded in Table 15. Many of these starches were so strongly degraded that, on repeated washing with water, small amounts were dissolved every time. The values of reducing power of the starches, therefore, are to some degree dependent on the intensity of the washing. Nevertheless, it can be seen that the reducing power increases in a regular way, and that the Lintner starch II:8 has an average chain length < 10.

The solutions of all Lintner starches of this series have a very low viscosity, the values varying around 1.3 cp for the 2 per cent solution.

All dissolved substances have low DP values. The filtrates from starches II:5–II:8 contain mostly glucose and disaccharides.

Microscopical examination

The granules of the first member of this series have a tolerably normal appearance. However, no striations are visible. The granules of Lintner starch II:2 already show a large number of deep fissures, half granules etc. In starch II:3 a certain number of granules is still visible; most of them, however, have cracked into many pieces. In the preparations II:5–II:8 nothing can be seen of the granule structure, only quite amorphous masses giving a weak reddish colour with iodine.

Table 15. Properties of Lintner starches and filtrates.

Series II.

Starch No.	Acid treatment, days	Yield, per cent	Reducing power, per cent glucose	Viscosity, 2 per cent solution, cp	Iodine reaction	
					D_{max}	λ_{max} mμ
II: 1	6	87	4.1	1.34	0.148	550
II: 2	12	78	3.6	1.45	0.137	545
II: 3	23	70	4.1	1.21	0.122	525
II: 4	37	64	5.1	1.29	0.103	520
II: 5	113	52	8.0	1.05	0.038	520
II: 6	144	50				
II: 7	152	—	9.4	1.25	0.028	510
II: 8	284	39	13.2	1.39		

Filtrates.

From starch No.	Yield, per cent	Reducing power, per cent "glucose"	Average chain length	$[\alpha]_D^{20°}$
II: 1	13.3	14.0	7.1	171°
II: 2	21.4	17.6	5.7	177°
II: 3	30.0	23.8	4.2	171°
II: 4	36.0	36.6	2.7	165°
II: 5	48.2	69.0	1.5	105°
II: 6	49.6	72.5	1.4	95°
II: 8	60.8	76.5	1.3	66°

Iodine reaction in solution

The density curves (Fig. 8) of the starch-iodine solutions were determined as described for series I. Maximum densities and wavelength of maximum absorption in Table 15. The colour intensities decrease strongly with increasing reducing power, and the maximum is shifted towards shorter wavelengths, *ca.* 500 mμ compared to *ca.* 620 mμ for native starch. The solutions of the most degraded Lintner starches give only a very weak brownish coloration.

Action of amylases on Lintner starches, series II

Lintner starch II: 1

The action of β-amylase was investigated: Table 16. The course of hydrolysis is quite normal and also the final yield of maltose, *ca.* 60 per cent.

Lintner starch II: 2

Starch. Action of malt α-amylase was determined: Table 16. The hydrolysis curve (curve 1 in Fig. 9 [●]) is normal. About 12 per cent of the glucosidic linkages are split in the dextrinization phase, as compared to about 17 per cent in the case of native starch.

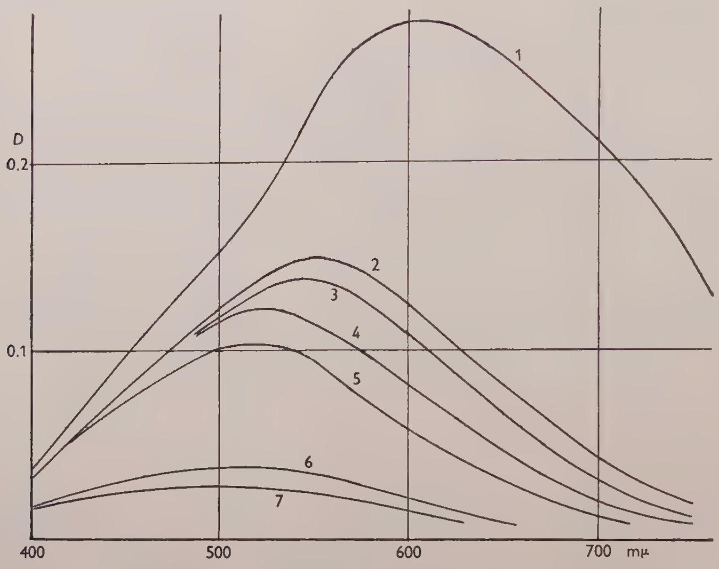


Fig. 8. Lintner starches and iodine. Optical densities at different wavelengths.

Curve 1; Native starch
2: Lintner starch II: 1
3: ,, ,, II: 2
4: ,, ,, II: 3
Curve 5: Lintner starch II: 4
6: ,, ,, II: 5
7: ,, ,, II: 7

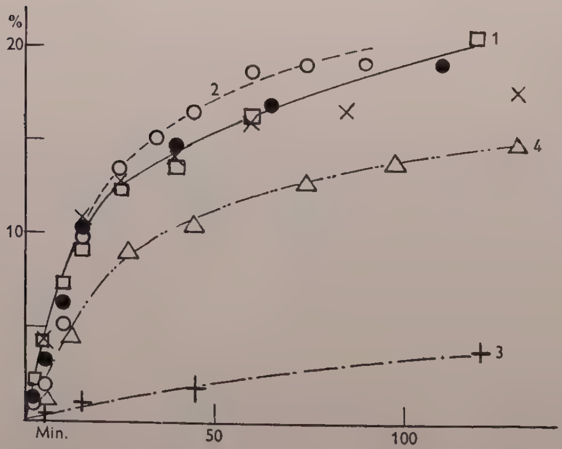


Fig. 9. Action of malt α-amylase.

Curve 1: Lintner starch II: 2.
2: ,, ,, II: 3.
3: Precipitate from acid solution, II: 3.
4. Lintner starch II: 7.

Table 16. Action of amylases on Lintner starches II: 1–II: 3.

Starch II: 1		Starch II: 2		Lintner starch II: 3			
β-Amylase		α-Amylase		Starch + α-amylase		Precipitate + α-amylase	
React. time	Hydrol., per cent	React. time	Hydrol., per cent	React. time	Hydrol., per cent	React. time	Hydrol., per cent
3 min.	2.6	2 min.	1.3	2 min.	0.9	5 min.	0.5
7 ,,	5.9	5 ,,	3.3	5 ,,	2.0	15 ,,	1.0
10 ,,	7.9	10 ,,	6.3	10 ,,	5.2	45 ,,	1.9
20 ,,	16.3	15 ,,	10.5	15 ,,	9.9	2 hrs.	3.8
40 ,,	24.5	40 ,,	14.5	25 ,,	13.5	3 ,,	4.0
60 ,,	26.3	65 ,,	16.9	35 ,,	15.1	21 ,,	6.9
2 hrs.	26.9	110 ,,	19.0	45 ,,	16.5	49 ,,	7.1
20 ,,	29.3	21 hrs.	29.6	60 ,,	18.5	74 ,,	7.1
44 ,,	31.2	46 ,,	34.3	75 ,,	19.0	92 ,,	7.1
		68 ,,	35.4	90 ,,	19.0		
		130 ,,	36.1	49 hrs.	42.3		
		153 ,,	37.8				

Filtrate. 100 ml filtrate was precipitated with ethanol: 4 g substance, or about 30 per cent, was recovered. Average DP = 13; no fermentable substances. The action of β-amylase on the precipitated fraction was investigated. When a constant value of the reducing power had been reached, fermentation experiments showed the presence of 65 per cent maltose and no glucose, i.e. approximately the normal result. Fig. 7 *A* may illustrate an average molecule of the precipitated fraction.

Lintner starch II: 3

Starch. The hydrolysis by malt α-amylase (Table 16, curve 2 [○] in Fig. 9) is normal; about 13 per cent of the linkages are ruptured in the rapid dextrinization phase.

Filtrate. Precipitation with ethanol yielded a fraction with average DP = 8. The action of malt α-amylase was determined: Table 16 and curve 3 (+) in Fig. 9 show that the rate of hydrolysis is very low compared to that of native starch or ordinary Lintner starch. This is in good agreement with our earlier results: the DP of the substrate is so low and, moreover, the substrate molecules so strongly branched, that the affinity of the enzyme to the substrate molecules is also low, and consequently a rapid dextrinization phase can hardly be observed. The slow increase of the reducing power is of the same kind as the slow saccharification of the primary α-dextrins observed in the hydrolysis of starch by malt α-amylase.

Lintner starch II: 4

Starch. Hydrolysis by α-amylase in Table 17 and curve 1 in Fig. 9 (□). The dextrinization phase comprises some 12 per cent of the linkages of the substrate.

Filtrate. The average DP of the ethanol-precipitated fraction was 5.0. Malt α-amylase had practically no action on this fraction in a reasonable period of time. This agrees well with our general conceptions of the enzyme action on starch degradation products. The DP of the substrate is so low, that the affinity to the enzyme must be

Table 17. Action of α-amylase on Lintner starches II: 4, 5 and 7.

Lintner starch II: 4		Lintner starch II: 5		Lintner starch II: 7	
Reaction time	Hydrolysis, per cent	Reaction time	Hydrolysis, per cent	Reaction time	Hydrolysis, per cent
2 min.	2.4	2 min.	2.2	5 min.	1.1
5 ,,	3.2	5 ,,	4.2	12 ,,	4.6
10 ,,	7.2	15 ,,	10.8	28 ,,	9.0
15 ,,	9.0	25 ,,	12.8	45 ,,	10.2
25 ,,	12.2	40 ,,	14.0	74 ,,	12.6
40 ,,	13.5	60 ,,	15.8	98 ,,	13.7
60 ,,	16.2	85 ,,	16.5	130 ,,	14.7
2 hrs.	20.4	130 ,,	18.6	22 hrs.	24.2
		52 hrs.	31.1		

very weak. Besides, since the 1,6-linkages are considerably more resistant to acid hydrolysis than are the 1,4-linkages, it must be concluded that in the dextrin fraction isolated from the acid extract the relative number of 1,6-linkages is higher than in starch and starch-like dextrins. This will decrease the enzyme-substrate affinity still more and may well make pentasaccharides practically stable to the enzyme action.

Lintner starch II: 5

Starch. Examination of the malt α-amylase action yielded a fairly normal result: Table 17 and Fig. 9 (×).

Filtrate. The average DP of the dissolved substances was 1.5, i.e. they consist mainly of glucose and disaccharides. Consequently only very little could be precipitated by addition of ethanol.

Lintner starch II: 7

The action of malt α-amylase upon this Lintner starch was found to be fairly normal. However, the dextrinization phase comprises only about 9 per cent of the substrate linkages: Table 17 and curve 4 in Fig. 9 (△).

Summary

Lintner starches have been prepared by treatment of native potato starch with 2 per cent or 10 per cent HCl, respectively, at 20°. The time of treatment was varied over a wide range. The starches have been characterized by their appearance under the microscope, their reducing power, iodine reaction, viscosity in 2 per cent solution, and by their behaviour when acted upon by amylases.

Prolonged treatment of the native starch with hydrochloric acid, especially with 10 per cent acid, results in the dissolution of a considerable portion of the starch substance and in a very marked hydrolysis of the long-chain molecules even *in* the remaining solid structure. For instance, the average chain length of the solid portion may be less than 10, and the granules may have turned completely into formless masses, but still the remains are practically insoluble in cold water (only very small amounts are leached out by intense washing), showing that the forces keeping the

chain molecules together in the starch granule may still be more or less undisturbed in spite of the hydrolysis of more than one glucosidic linkage in ten.

The dissolution of substance from the granules may start simultaneously in more or less amorphous regions throughout the whole granule. In any case, it seems that dissolution begins immediately at the surface since the concentric striations of the native granules disappear very rapidly. The granules do not swell appreciably in the acid, provided its concentration does not exceed *ca.* 12 per cent.

Since fractions of an average DP > 10 have been isolated from the acid filtrates, it is obvious that in the dissolution process relatively large portions of chain molecules are released. It seems safe to assume that dissolution of this kind takes place from the less oriented parts of the granule, whereas the highly organized, "micelle"-like regions are more resistant to the action of the acid.

Prolonged action of the acid naturally causes hydrolysis of dissolved saccharides, so that after very long periods chiefly glucose and disaccharides are present.

The colour reaction of the Lintner starches with diluted iodine solution changes with increasing reducing power of the starch from dark blue to a weak brownish red. This corresponds to a displacement of the absorption maximum from about 620 mμ to about 500 mμ. The colour intensity diminishes regularly with decreasing chain length. No colour is given by open-chain dextrin fractions of an average DP less than about 10 units.

The starch granules absorb iodine from iodine solutions. However, the amount of bound iodine is rather small, which is probably explained by the fact that the starch molecules in the granules are fixed radially in a more or less extended state. The helical arragement of the chains, which is a prerequisite for the binding of large amounts of iodine, does not occur in the granules.

The action of amylases on the various Lintner starches, even those of rather small average chain length, is substantially the same as that on native starch solutions. This confirms the opinion that the extent of enzymatic conversion and the kinetics of the enzymatic hydrolysis depend chiefly on the chemical structure of the substrate, especially on its degree of ramification, and only to a minor extent on the physical state of the substrate solutions. It shall not be denied, however, that in certain cases, and particularly when amylose is the substrate, "retrogradation" of the substrate may play a role in its susceptibility to the enzymatic attack.

The action of malt α-amylase on native starch comprises a rapid dextrinization, i.e. hydrolysis of about 17 per cent of the glucosidic linkages of the starch, after which follows a slow saccharification, more or less complete. The number of linkages ruptured in the dextrinization phase is different for different substrates, e.g. about 22 per cent for amylose, about 10 per cent for glycogen. These facts and, in general, the mechanism of the malt α-amylase action, are explained as depending chiefly on the degree of ramification of the substrate and the mode of attachment enzyme-substrate. Lintner starches, exhibiting a moderate reducing power, behave as native starch. Qualitatively, the same result is found also with Lintner starches of very high reducing power. However, when the average DP of the starch is very low, $<$ *ca.* 20 units, the relative number of linkages ruptured in the rapid dextrinization phase begins to decrease with decreasing DP and finally, when DP is $<$ *ca.* 5, no rapid phase can be observed at all. This is explained by the theory proposed by one of the authors concerning the attachment of malt α-amylase to the substrates.

Institute of Organic Chemistry and Biochemistry, University, Stockholm.

REFERENCES

1. Lintner, C. J., J. prakt. Chem. *39*, 378 (1886).
2. Kerr, R. W., Chemistry and industry of starch, 2nd Ed., Acad. Press, New York 1950.
3. Berner, E. and Petersen, R., Ber. *65*, 687 (1932); Berner, E. and Melhus, F., Ber *66*, 1333 (1933).
4. Summarized in Myrbäck, K. and Neumüller, G., in Sumner, J. B. and Myrbäck, K., The Enzymes, Acad. Press, New York 1950, Vol. I, p. 653; Myrbäck, K., Advances Carbohydrate Chem. *3*, 251 (1948).
5. Myrbäck, K. and Thorsell, W., Svensk Kem. Tidskr. *55*, 178 (1943); Myrbäck, K., Stenlid, G., and Nycander, G., Biochem. Z. *316*, 433 (1944); Carlqvist, B., Acta Chem. Scand. *2*, 770 (1948); Myrbäck, K., Arkiv Kemi, Mineral. Geol. *26 A*, No. 7 (1948).
6. Myrbäck, K. and Sillen, L. G., Svensk Kem. Tidskr. *56*, 60 (1944); Sillen, L. G. and Myrbäck, K., Ibid. *56*, 142 (1944); Myrbäck, K., The Svedberg 1884–1944, Uppsala–Stockholm 1944, p. 474; Myrbäck, K. and Johansson, N. O., Arkiv Kemi, Mineral. Geol. *20 A*, No. 6 (1945); Myrbäck, K., Arch Biochem. *14*, 53 (1947); Myrbäck, K., Arkiv Kemi *2*, 417 (1950).
7. Meyer, K. H. and Gonon, W. F., Helv. Chim. Acta *34*, 294 (1951).
8. Myrbäck, K., Biochem. Z. *307*, 140 (1941).
9. Myrbäck, K., Hoppe-Seylers Z. physiol. Chem. *139*, 30 (1924).
10. Meyer, K. H. and Bernfeld, P., Helv. Chim. Acta *25*, 399 (1942).
11. Myrbäck, K., Arkiv Kemi *4*, 433 (1952).

Tryckt den 9 juni 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 35

Communicated 10 February 1954 by ARNE TISELIUS and GUNNAR HÄGG

On the properties of binary mixtures

I. Influence of compound formation on activity factors and activities

By ERIK HÖGFELDT

With 16 figures in the text

Introduction

By assuming chemical reactions between the components DOLEZALEK (1908) was the first to try to explain deviations from Raoult's law in binary mixtures. He assumed the ideal laws to be valid, the deviations being ascribed to the fact that the true amounts of the components had not been taken into consideration. Denoting the components by A and B, he developed equations for the two cases: (1) the formation of the compound AB and (2) the association of A to A_2. For compound formation the activities showed negative and, for association, positive deviations from Raoult's law. DOLEZALEK and his coworkers obtained surprisingly good agreement between calculated and experimentally obtained vapour pressures for several liquid, binary mixtures.

Later on HILDEBRAND and EASTMAN (1915) developed expressions for calculating the vapour pressure of thallium amalgams by assuming the formation of $TlHg_2$.

Recently METZGER and SAUERWALD (1950) have given activity curves on the same assumptions, for certain simple values for u und v, where u and v give the composition of the compund $A_u B_v$. They also point out, that the deviations from Raoult's law are *both* positive and negative for all types of compounds except AB.

As was clearly stated by WASHBURN (1912), no assumption of chemical reactions could explain the formation of two liquid phases, if the ideal laws were retained. For that reason the assumption of chemical reactions as the sole possibility of explaining deviations from ideality was abandoned and other treatments which take e.g. the van der Waals forces into consideration have been developed. (See for instance HILDEBRAND-SCOTT [1950].)

DOLEZALEK completely neglected the van der Waals forces in his treatment of binary mixtures but in recent years there has been, as pointed out by REDLICH (1949), a tendency to forget the possibility of chemical reactions when discussing binary mixtures. REDLICH also points out the difficulties of distinguishing between the chemical interaction explanation and other concepts. As there may be systems, where these effects are predominating over the van der

Waals and other effects, the influence of chemical interactions on the thermodynamic functions must be known if we shall be able to recognize them.

For that reason the influence of compound formation on activity factors and activities will now be discussed more completely than in the papers cited above. The general case for the formation of aggregates $(A_u B_v)_n$ will be considered. In addition, the case for the building up of compounds AB_n by addition of B to A is also studied.

Some of the results below have already been published in a short communication, HÖGFELDT (1951).

I. Compounds $(A_u B_v)_n$ can be formed

Basic assumptions and relations. We shall follow DOLEZALEK and assume: the components A and B are completely miscible at all concentrations and interact to form the compounds $(A_u B_v)_n$ and the ideal laws are valid for each of the species A, B and $(A_u B_v)_n$ in the mixture.

For the sake of simplicity we have selected from the infinite number of possibilities those compounds that are formed by association of $A_u B_v$ to larger aggregates.

Other possibilities of deviations from ideal behaviour, e.g. the van der Waals forces, are neglected in order to clarify the influence of compound formation on activity factors and activities, although in practice such a treatment will be only a limiting case.

The compounds A and B interact to form the compounds $(A_u B_v)_n$ according to the following series of reactions:

$$\left.\begin{array}{lll} uA + vB & \rightleftharpoons & A_u B_v \\ x_A \qquad x_B & & y_1 \\ A_u B_v + A_u B_v & \rightleftharpoons & (A_u B_v)_2 \\ y_1 \qquad\quad y_1 & & y_2 \\ \vdots \qquad\quad \vdots & & \vdots \\ A_u B_v + (A_u B_v)_{p-1} & \rightleftharpoons & (A_u B_v)_p \\ y_1 \qquad\quad y_{p-1} & & y_p \end{array}\right\} \quad (1) \qquad \left.\begin{array}{l} k_1 = \dfrac{y_1}{x_A^u \cdot x_B^v} \\ k_2 = \dfrac{y_2}{y_1^2} \\ \vdots \\ k_p = \dfrac{y_p}{y_1 \cdot y_{p-1}} \end{array}\right\} \quad (2)$$

where x_A and x_B are the mole fractions of A and B and $y_1, y_2 \ldots y_p$ are the mole fractions of the compounds $A_u B_v$, $(A_u B_v)_2 \ldots (A_u B_v)_p$. These mole fractions are called the true mole fractions as distinguished from the apparent mole fractions defined below. The constants $k_1, k_2 \ldots k_p$ are the equilibrium constants of reactions (1) and defined by the expressions (2).

As we have used mole fractions for the components in the mixture we have the following relation:

$$x_A + x_B + \Sigma\, y_n = 1, \tag{3}$$

where the symbol Σ indicates summation over the values $1, 2, \ldots n \ldots p$. Using the equilibrium constant products $q_1, q_2, \ldots q_n$ defined by:

$$q_n = k_1^n \cdot k_2 \ldots k_n \tag{4}$$

we obtain the following expression for y_n:

$$y_n = k_2 \dots k_n \cdot y_1^n = q_n \cdot x_A^{nu} \cdot x_B^{nv}. \tag{5}$$

The apparent mole fractions of A and B, i.e. those obtained by determination of the total amounts of A and B, are connected with the true mole fractions by:

$$\left.\begin{aligned} X_A &= (x_A + u \,\Sigma\, n y_n)/(x_A + x_B + (u+v)\,\Sigma\, n y_n) \\ X_B &= (x_B + v \,\Sigma\, n y_n)/(x_A + x_B + (u+v)\,\Sigma\, n y_n) \end{aligned}\right\} X_A + X_B = 1. \tag{6}$$

Since the ideal laws are assumed to be valid, we have the following expressions for the activities a_A, a_B and activity factors γ_A, γ_B:

$$\left.\begin{aligned} a_A &= x_A \\ a_B &= x_B \end{aligned}\right\} \quad (7) \qquad \left.\begin{aligned} \gamma_A &= x_A/X_A \\ \gamma_B &= x_B/X_B \end{aligned}\right\} \quad (8)$$

when taking the pure component as the standard states.

Using equations (2) ... (8) we can calculate the shapes of the activity and activity factor curves for different values of u, v and k_1, $k_2 \dots k_p$.

It may be noted that relations of the type (6) have been used by REDLICH and KISTER (1947) when discussing linear association equilibria.

The activity factor curves

In Figs. 1 and 2 γ_A and γ_B are plotted against X_B assuming in Fig. 1 the formation of the compound A_3B only and in Fig. 2 the formation of only A_2B_3. The activity factors have been calculated for both $k_1 = 1$ and $k_1 = 10$ using equations (2) ... (8) for $n = 1$.

From Fig. 1 it is seen that γ_A and γ_B intersect each other at $X_B = \frac{1}{4}$ independent of the value of k_1. The limiting values are 1 with the exception of γ_B for $X_B = 0$, which has a limiting value different from 1 and dependent upon k_1. γ_B has a maximum and γ_A a minimum at $X_B = \frac{1}{2}$. That γ_A and γ_B have extremes, one a maximum and the other a minimum, for the same X_B-value follows from Gibbs-Duhem's equation, which is valid even if compounds are formed as is discussed below. From Fig. 2 we find that $\gamma_A = \gamma_B$ for $X_B = 0.6$, that all limiting values are 1, and that each activity factor curve has two extremes.

These observations are special cases of general rules, which we shall now derive.

Using equations (5), (6) and (8) we obtain the following expressions for γ_A and γ_B:

$$\left.\begin{aligned} \gamma_A &= (x_A + x_B + (u+v)\,\Sigma\, n q_n \cdot x_A^{nu} \cdot x_B^{nv})/(1 + u \,\Sigma\, n q_n \cdot x_A^{nu-1} \cdot x_B^{nv}) \\ \gamma_B &= (x_A + x_B + (u+v)\,\Sigma\, n q_n \cdot x_A^{nu} \cdot x_B^{nv})/(1 + v \,\Sigma\, n q_n \cdot x_A^{nu} \cdot x_B^{nv-1}) \end{aligned}\right\} \tag{9}$$

The point of intersection. By setting $\gamma_A = \gamma_B$ in (9) we find:

$$u x_B = v x_A.$$

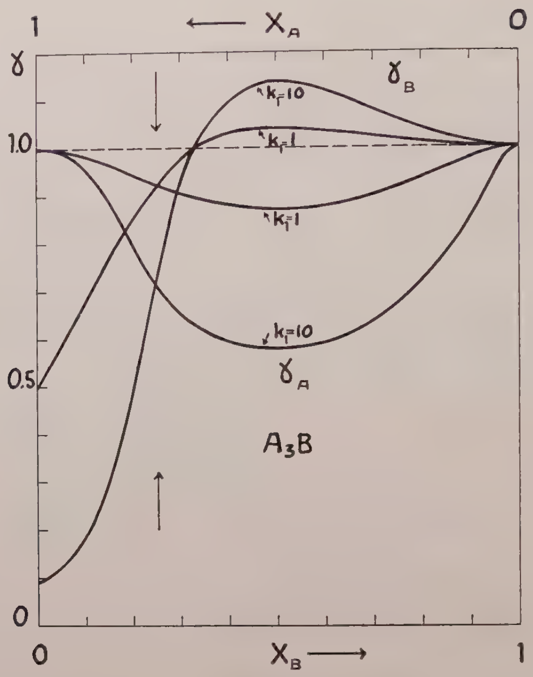


Fig. 1. The activity factors γ_A and γ_B are plotted against X_B, assuming the formation of A_3B with $k_1 = 1$ and $k_1 = 10$. The arrows indicate the point of intersection of the activity factor curves at $X_B = 0.25$. The straight line $\gamma = 1$ is dashed.

which inserted in (8) gives:

$$X_B = v/(u+v). \tag{10}$$

Note: In the following only the X_B-values for points of interest have been calculated. X_A-values follow from $X_A = 1 - X_B$ (6).

The position of the point of intersection between γ_A and γ_B is thus only a function of the composition of the compounds and permits us to find the ratio of u to v. To find the absolute values of u and v one more relation is necessary. Relations which permit this determination, at least for single-compound systems, are developed below. In Fig. 1 the point of intersection at $X_B = \frac{1}{4}$ gives $u = 3v$ in agreement with the assumed compound A_3B. In Fig. 2 the point of intersection is situated at $X_B = 0.6$ in agreement with the formula A_2B_3. The relation (10) can also be verified from Fig. 3 for the system $AB + (AB)_2$ and in Fig. 4 for $(AB)_{n\to\infty}$ where $(AB)_{n\to\infty}$ means that AB associates to infinitely large aggregates.

To Figs. 4 and 8: For evaluating $\Sigma\, y_n$ for an infinite number of compounds some simplifying assumptions concerning the values of $k_1, k_2, \ldots k_p$ must be made. In Figs. 4 and 8, the following two assumptions have been used: a) $k_1 = k_2 = \cdots = k_p = k = 1$, and b) $k_2 = k_3 = \cdots = k_p = k = 10$ and $k \neq k_1 = 1$. Because of these two assumptions $\Sigma\, y_n$ becomes a simple geometric series which is easy to sum. The term $\Sigma\, n\, y_n$ is then obtained by differentiation of $\Sigma\, y_n$.

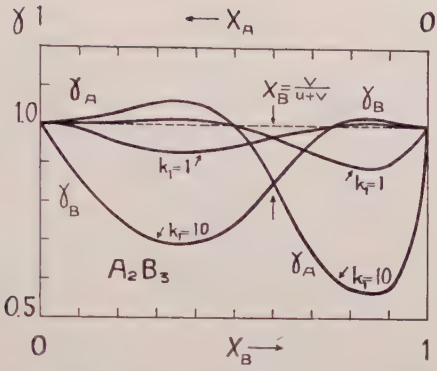


Fig. 2. The activity factors γ_A and γ_B are plotted against X_B, assuming the formation of A_2B_3 with $k_1 = 1$ and $k_1 = 10$. The arrows indicate the point of intersection of the activity factor curves at $X_B = 0.6$. The straight line $\gamma = 1$ is dashed.

The point of intersection with the line $\gamma = 1$. In Figs. 1 and 2 the straight line $\gamma = 1$ has been dotted. For its points of intersection with γ_A and γ_B we can derive from (2) ... (8):

$$\left.\begin{array}{l} \gamma_A = 1;\quad a_A = X_A;\quad X_B = (\bar{n}v - 1)/(\bar{n}(u+v) - 1) \\ \gamma_B = 1;\quad a_B = X_B;\quad X_B = \bar{n}v/(\bar{n}(u+v) - 1) \end{array}\right\} \tag{11}$$

where the number average $\bar{n}$ is defined by:

$$\bar{n} = \Sigma\, n y_n / \Sigma\, y_n = \Sigma\, n q_n \cdot x_A^{nu} \cdot x_B^{nv} / \Sigma\, q_n \cdot x_A^{nu} \cdot x_B^{nv}. \tag{12}$$

This number average gives the average number of units $A_u B_v$ in the compounds $(A_u B_v)_n$ and has thus to be distinguished from the number average used in complex chemistry which gives the average number of ligands bound per central atom.

From (11) and the points of intersection with $\gamma = 1$, it is possible to calculate u and v only for single-compound systems ($\bar{n} = 1$). For multi-compound systems this is not so simple as $\bar{n}$ is in most cases an unknown function of the composition.

From Fig. 1 we find that γ_B intersects $\gamma = 1$ at $X_B = \frac{1}{3}$ and that γ_A has no point of intersection. By inserting the values $X_B = 0$ and $X_B = \frac{1}{3}$ in (11) and using $\bar{n} = 1$ one finds $u = 3$, $v = 1$. In the same manner the formula A_2B_3 can be derived from Fig. 2 using (11). These examples show how one can determine whether an experimentally obtained activity factor curve may be explained by compound formation or not.

It is important to stress that the relations (10), (11) and (16) below are valid even if some compounds are missing, i.e. some $k_n = 0$, as is easily seen from the derivation of these expressions. They are thus of general validity, the only conditions apart from the ideal laws to be fulfilled being that the aggregates are built up of units $A_u B_v$ where u and v are constants.

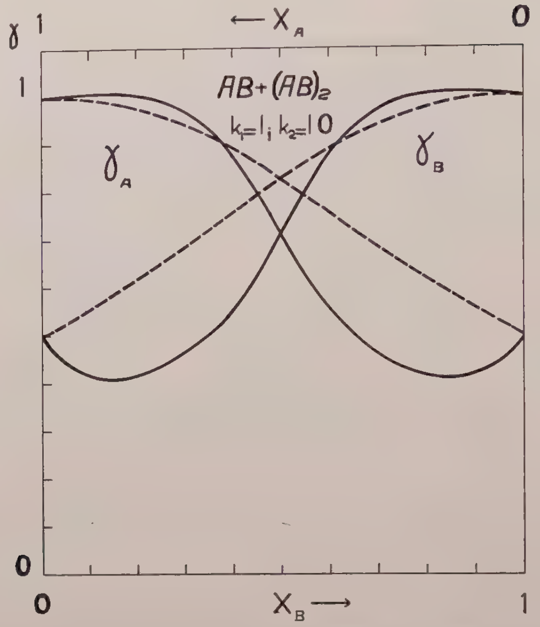


Fig. 3. The activity factors γ_A and γ_B are plotted against X_B, assuming the formation of the compounds $AB + (AB)_2$ with $k_1 = 1$ and $k_2 = 10$. For the sake of comparison the activity factors for the formation of only AB with $k_1 = 1$ are dashed.

The extremes. It has not been possible to find any simple relation for the positions of the extremes for the general case, but for single-compound systems a relation was found as follows:

The following two functions were studied:

$$f(x_A, x_B) = \gamma_A/\gamma_B = (1 + v\,k_1\,x_A^u \cdot x_B^{v-1})/(1 + u\,k_1\,x_A^{u-1} \cdot x_B^v), \tag{13}$$

$$\varphi(x_A, x_B) = 1 - x_A - x_B - k_1\,x_A^u \cdot x_B^v = 0. \tag{14}$$

By differentiating (13) and (14), setting $d f(x_A, x_B) = 0$ and eliminating $d x_A / d x_B$ we find, using (3) ... (6) for $n = 1$, an expression which can be simplified to:

$$X_B = \frac{1}{u+v}\left[v \pm \sqrt{\frac{u\,v}{u+v-1}}\right] \tag{15}$$

where the minus sign applies to the maximum of γ_A and the plus sign to the maximum of γ_B.

For single-compound systems the positions of the extremes are thus only functions of u and v and determined by (15). For multi-compound systems it is felt that $\bar{n}$ appears as a third variable in the expression analogous to (15) and thus u and v will not be as easily determined as from (15).

Concerning the number of extremes for multi-compound systems, the relations (11) and (16) below show that γ_A and γ_B must, for $u > 1$ and $v > 1$, intersect

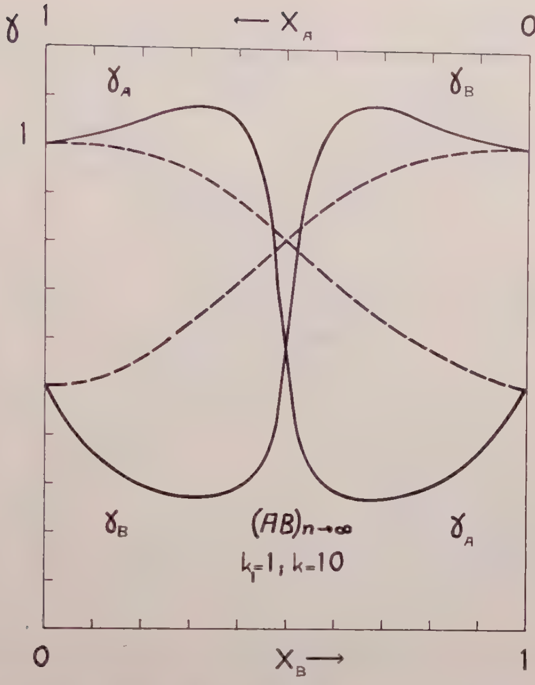


Fig. 4. The activity factors γ_A and γ_B are plotted against X_B, assuming the formation of an infinite number of compounds of the composition $(AB)_n$, denoted by $(AB)_{n\to\infty}$, with $k_1 = 1$ and $k_2 = k_3 = \cdots = k = 10$. For the sake of comparison the activity factors for the formation of $(AB)_{n\to\infty}$ with $k_1 = k_2 = \cdots = k = 1$ are dashed.

the straight line $\gamma = 1$ in three points and must therefore have at least *two* extremes each.

The relations (15) can be verified from Figs. 1 and 2 where the extremes are situated at the points corresponding to the assumed compounds A_3B and A_2B_3.

From Figs. 1 and 2 it is seen that γ_A has a maximum when γ_B has a minimum and vice versa. This is one consequence of Gibbs-Duhem's equation, which is valid also if compounds are formed, as may be seen from the following argument:

By differentiation of (3) after inserting the value of y_n from (5) we get:

$$dx_A(1 + u\,\Sigma\, n q_n x_A^{nu-1} \cdot x_B^{nv}) + dx_B(1 + v\,\Sigma\, n q_n x_A^{nu} \cdot x_B^{nv-1}) = 0$$

which can be transformed to:

$$dx_A/dx_B = -(1 + v\,\Sigma\, n q_n x_A^{nu} \cdot x_B^{nv-1})/(1 + u\,\Sigma\, n q_n x_A^{nu-1} \cdot x_B^{nv}) = -\gamma_A/\gamma_B = \\ = -x_A/X_A/x_B/X_B$$

which gives:

$$X_A x_B \cdot dx_A + X_B x_A \cdot dx_B = X_A \cdot dx_A/x_A + X_B \cdot dx_B/x_B = X_A\, d\ln x_A + X_B\, d\ln x_B = 0.$$

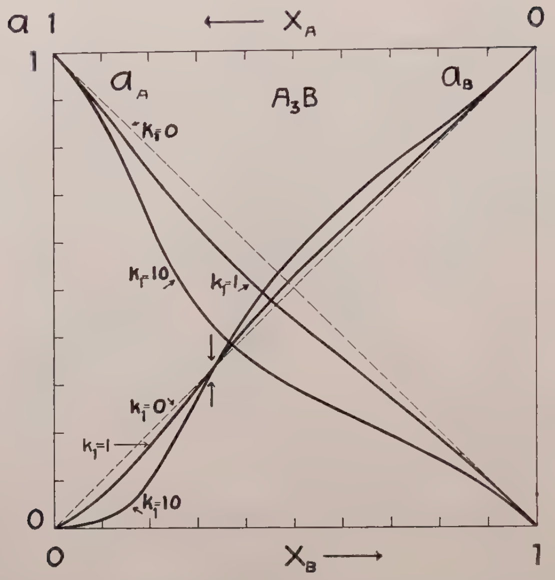


Fig. 5. The activities a_A and a_B are plotted against X_B, assuming the formation of A_3 B with $k_1=1$ and $k_1=10$. The dashed straight lines give a_A and a_B if Raoult's law were valid, i.e. $k_1=0$. The arrows at $X_B=0.33$ indicate the point where a_B changes from positive to negative deviations from Raoult's law.

This expression is Gibbs-Duhem's equation since $a_A=x_A$ and $a_B=x_B$. Gibbs-Duhem's equation is also valid for the general case if compounds A_p B_q are formed where p and q are variables which can be shown in the same manner as above.

The limiting values. From (9) we obtain the following limiting values for γ_A and γ_B:

$$\left.\begin{array}{l} X_B=0;\ (x_A=1,\ x_B=0);\ \gamma_A=1;\ \gamma_B=1 \text{ for } v>1,\ \gamma_B=1/(1+k_1) \text{ for } v=1 \\ X_B=1;\ (x_A=0,\ x_B=1);\ \gamma_B=1;\ \gamma_A=1 \text{ for } u>1,\ \gamma_A=1/(1+k_1) \text{ for } u=1 \end{array}\right\} \quad (16)$$

The expressions (16) permit us to determine the value of k_1 from the limiting values of γ_A and γ_B if $u=1$ or $v=1$. For single-compound systems this is the only equilibrium constant to be determined. For multi-compound systems there are a number of constants to be determined and methods for finding them will be discussed in connection with the testing of the assumption of compound formation on experimentally studied binary mixtures.

In Fig. 1 where γ_B has the limiting values, 0.5 and 0.09, the values $k_1=1$ and $k_1=10$ can be verified using (16). Where both $u>1$ and $v>1$ as in Fig. 2, all limiting values are equal to unity and we cannot draw any conclusions concerning the value of k_1 from them. In Figs. 3 and 4 the value of $k_1=1$ can be verified for the systems $AB+(AB)_2$ and $(AB)_{n\to\infty}$ using (16).

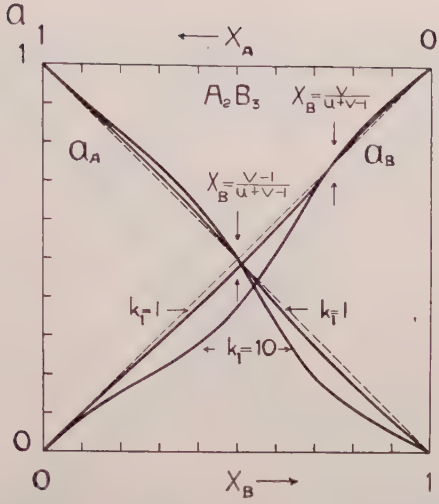


Fig. 6. The activities a_A and a_B are plotted against X_B, assuming the formation of A_2B_3 with $k_1 = 1$ and $k_1 = 10$. The dashed straight lines give a_A and a_B if Raoult's law were valid. The arrows at $X_B = 0.5$ and $X_B = 0.75$ indicate the points where the activities change from positive to negative deviations from Raoult's law.

The activity curves

In Figs. 5 and 6 the activity curves are plotted against X_B, assuming in Fig. 5 the formation of the compound A_3B and in Fig. 6 the formation of A_2B_3. The activities have been calculated for both $k_1 = 1$ and for $k_1 = 10$ using equations (2) ... (8) for $n = 1$.

In Figs. 5 and 6 we find that a_A and a_B can have both positive and negative deviations from Raoult's law. In Fig. 5 only a_B has both deviations but in Fig. 6 both a_A and a_B have positive and negative deviations. It is also seen that the position of the point of intersection of a_A and a_B is dependent upon k_1.

Note: When here and in the following reference is made to Raoult's law, the form is aimed at where the activity is equal to the apparent mole fraction.

The points of intersection with the lines $a_A = X_A$ and $a_B = X_B$. The points where the deviations are changing from positive to negative are characterized by $\gamma = 1$. For the points of intersection of a_A and the straight line $a_A = X_A$ and of a_B and $a_B = X_B$ we thus get the relations (11) previously derived:

$$\left.\begin{aligned}\gamma_A = 1;\ a_A = X_A;\ X_B = (\bar{n}v - 1)/(\bar{n}(u+v) - 1)\\ \gamma_B = 1;\ a_B = X_B;\ X_B = \bar{n}v/(\bar{n}(u+v) - 1)\end{aligned}\right\} \qquad (11)$$

From (11) we find that, for $u = v = 1$, we have no points of intersection and that the deviations must according to (3) and (6) be negative as was found by DOLEZALEK (1908). For all other cases we must have both positive and negative deviations as pointed out by METZGER and SAUERWALD (1950).

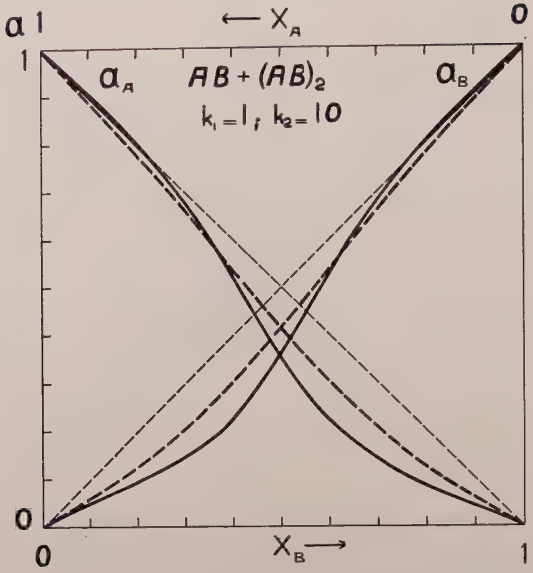


Fig. 7. The activities a_A and a_B are plotted against X_B, assuming the formation of the compounds $AB+(AB)_2$ with $k_1=1$ and $k_2=10$. For the sake of comparison the activities for the formation of only AB with $k_1=1$ are dashed. The dashed straight lines give a_A and a_B if Raoult's law were valid.

In Figs. 5 and 6 the positions of the points where the deviations are changing from positive to negative permit us to determine u and v in a manner analogous to that made for the activity factor curves. The composition of the compound in single-compound systems can thus be equally well determined from the activity as from the activity factor curves. In Figs. 7 and 8, where the activities have been plotted for the two systems: $AB+(AB)_2$ and $(AB)_{n\to\infty}$ it is seen that the sign of the deviation alters at the same X_B-values as those at which the activity factor curves in Figs. 3 and 4 intersect the line $\gamma=1$, in agreement with (11).

The point of intersection. By setting $a_A=a_B$ no simple relation like (10) could be found since the position of this point of intersection is dependent upon the values of $k_1, k_2 \ldots k_p$. But, because of the symmetry of the functions (5) ... (8), the activity and activity factor curves for $u=v$ are symmetric about $X_B=0.5$ and the curves for A_nB_m are the mirror images of those for A_mB_n. From these considerations we conclude that, when $u=v$, a_A and a_B must intersect each other at $X_B=0.5$. This is also easily shown by setting $a_A=a_B$ in (7) ... (9). But only in this special case is the position of this point independent of the values of $k_1, k_2 \ldots, k_p$.

The mole fraction of A_uB_v

For single-compound systems the mole fraction of the compound (y_1) is obtained by setting $n=1$ in (3). In Fig. 9, y_1 has been plotted against X_B assuming the formation of some different compounds. The value of the equi-

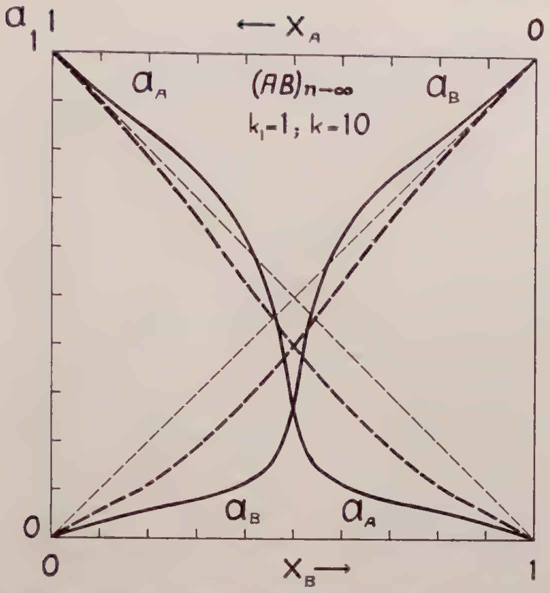


Fig. 8. The activities a_A and a_B are plotted against X_B, assuming the formation of an infinite number of compounds of the composition $(A\,B)_n$, denoted by $(A\,B)_{n\to\infty}$, with $k_1 = 1$ and $k_2 = k_3 = \cdots = k = 10$. For the sake of comparison the activities for the formation of $(A\,B)_{n\to\infty}$ with $k_1 = k_2 = \cdots = k = 1$ are dashed. The dashed straight lines give a_A and a_B if Raoult's law were valid.

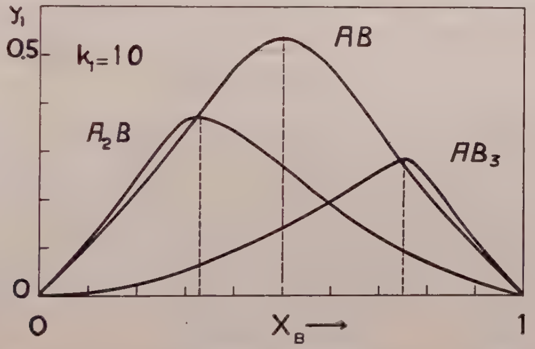


Fig. 9. The mole fraction (y_1) of $A_u\,B_v$ in some single-compound systems is plotted against X_B for the following compounds: $A\,B$, $A_2\,B$ and $A\,B_3$ with $k_1 = 10$. The dashed vertical lines indicate the maxima situated at the points corresponding to the composition of the compound.

librium constant k_1 has been set equal to 10. From Fig. 9 we find that y_1 has a maximum at the point determined by (10), corresponding to the composition of the compound. By differentiating (3) setting $dy_n = 0$ and using $dx_A + dx_B = 0$ when $dy_n = 0$, this not unexpected observation is easily verified for every y_n independent of the value of n.

The number average

In Fig. 10 $\bar{n}$ as defined by (12) is given as a function of X_B for some different systems. That $\bar{n}$ has a maximum at the point determined by (10) can

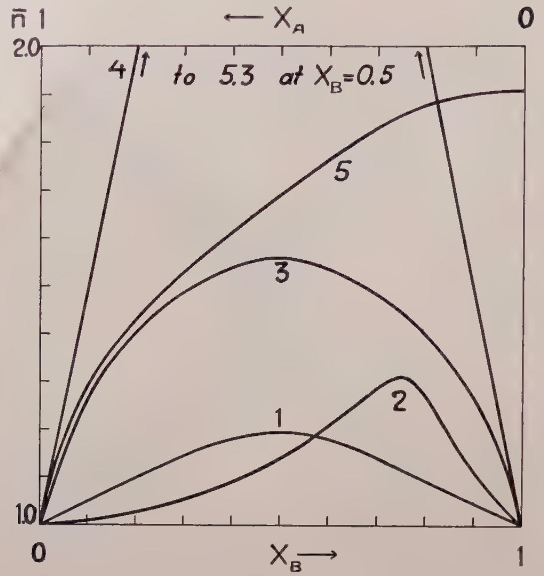


Fig. 10. The number average $\bar{n}$ is plotted against X_B for the following systems: 1) $(A\,B)_{n\to\infty}$, $k_1 = k_2 = \cdots = k = 1$; 2) $(A\,B_3)_{n\to\infty}$, $k_1 = k_2 = \cdots = k = 1$; 3) $A\,B + (A\,B)_2$, $k_1 = 1$, $k_2 = 10$; 4) $(A\,B)_{n\to\infty}$, $k_1 = 1$, $k_2 = k_3 = \cdots = k = 10$; 5) $A\,B + A\,B_2$, $k_1 = 1$, $k_2 = 10$.

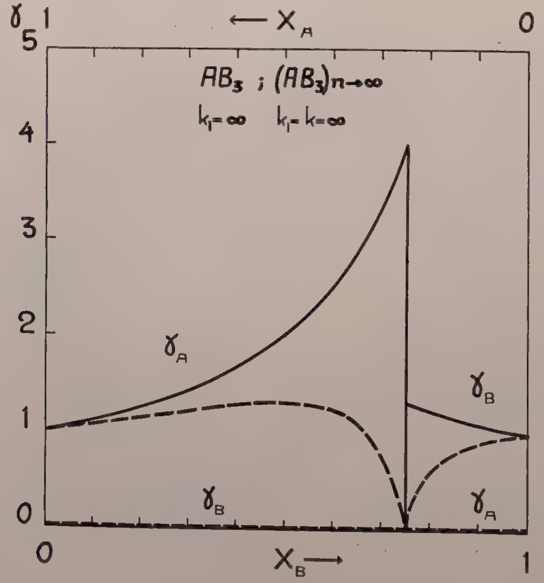


Fig. 11. The activity factors γ_A and γ_B are plotted against X_B for the two limiting cases: $A\,B_3$, $k_1 = \infty$ (dashed curves), and $(A\,B_3)_{n\to\infty}$, $k_1 = k_2 = \cdots = k = \infty$ (full-drawn curves).

be shown by differentiation of (12) in a manner analogous to the derivation for the position of the maximum of y_n:

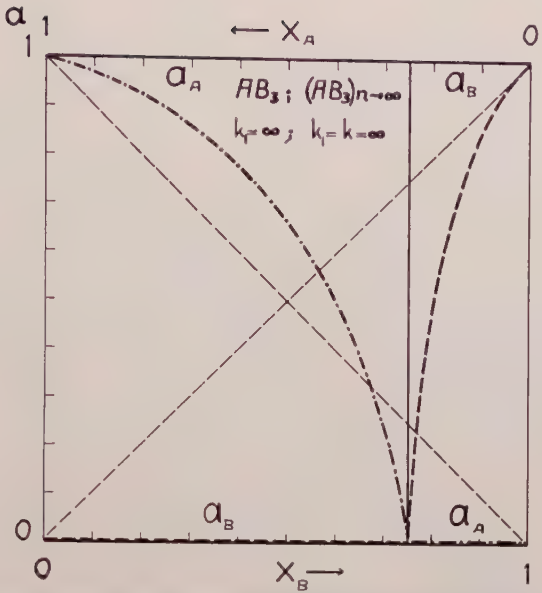


Fig. 12. The activities a_A and a_B are plotted against X_B for the two limiting cases: A B_3, $k_1 = \infty$ (dashed curves), and $(A\,B_3)_{n\to\infty}$, $k_1 = k_2 = \cdots = k = \infty$ (full-drawn curves). The last-mentioned activities are either 1 or 0 changing at $X_B = 0.75$. The dashed straight lines give a_A and a_B if Raoult's law were valid.

For the case of infinite association with $k_2 = k_3 = \cdots = k$ and $k = k_1$ or $k \neq k_1$, it can be shown that:

$$\bar{n} < 1 + k. \tag{17}$$

For instance, if $k = 1$, $\bar{n}$ is always less than 2, which means that the aggregation of the molecules $A_u\,B_v$ is rather insignificant. By comparing the dashed functions in Figs. 3, 4, 7 and 8, it is seen how little the activities and activity factors for $(A\,B)_{n\to\infty}$, $k_1 = k = 1$, deviate from the same curves for AB, $k_1 = 1$.

When comparing the heights of the maxima of the curves, 1, 2 and 4 with the values given by (17), it is found that they are much less than the maximum values given by (17).

Infinite constants

Single-compound systems. In order to discuss more thoroughly the influence of k_1, the limiting case $k_1 = \infty$ will also be studied. $k_1 = \infty$ implies that, on mixing A and B, all molecules that possibly can react form the compound $A_u\,B_v$. This fact makes the calculations quite simple. By adding B to an excess of A and using equations (3), (5) and (6) for $n = 1$, we obtain:

$$a_A = x_A = (v - (u+v)\,X_B)/(v - (u+v-1)\,X_B) \tag{18}$$

$$a_B = x_B = 0;\quad y_1 = 1 - x_A$$

From (8) and (18):

$$\gamma_A = (v - (u+v)\,X_B)/(1 - X_B)\cdot(v - (u+v-1)\,X_B)$$

$$\gamma_B = 0 \tag{19}$$

valid for $0 \leq X_B \leq v/(u+v)$

By adding A to an excess of B we find in the same manner:

$$\left.\begin{aligned} a_{\mathrm{A}} &= x_{\mathrm{A}} = 0;\ y_1 = 1 - x_{\mathrm{B}} \\ a_{\mathrm{B}} &= x_{\mathrm{B}} = ((u+v)\,X_{\mathrm{B}} - v)/((u+v-1)\,X_{\mathrm{B}} + 1 - v) \\ \gamma_{\mathrm{A}} &= 0 \\ \gamma_{\mathrm{B}} &= ((u+v)\,X_{\mathrm{B}} - v)/X_{\mathrm{B}} \cdot ((u+v-1)\,X_{\mathrm{B}} + 1 - v) \end{aligned}\right\}\ \begin{array}{l} \text{valid for} \\ v/(u+v) \le X_{\mathrm{B}} \le 1 \end{array} \qquad \begin{array}{r} (20) \\ \\ \\ (21) \end{array}$$

In Figs. 11 and 12 the dashed activity factor and activity curves are calculated for AB_3, $k_1 = \infty$. Of course, equations (10), (11) and (15), which are independent of k_1, are also valid in this case as is seen in Figs. 11 and 12 and can be verified from (18)...(21).

It is interesting to note the low maximum value of γ_{A} (~ 1.33). By inserting the appropriate values of X_{B} from (15) into (19) and (21) we obtain:

$$\left.\begin{aligned} \gamma_{\mathrm{A\ max.}} &= \left[(u+v)/(\sqrt{v} + \sqrt{u\,(u+v-1)})\right]^2 \\ \gamma_{\mathrm{B\ max.}} &= \left[(u+v)/(\sqrt{u} + \sqrt{v\,(u+v-1)})\right]^2 \end{aligned}\right\} \qquad (22)$$

For $u = 1$, $v = 3$ we get $\gamma_{\mathrm{A\ max.}} = \frac{4}{3} = 1.33$.

From (8) and (15) we find that $\gamma_{\mathrm{A}}(k_1)$ has extremes at the same points as $x_{\mathrm{A}}(k_1)$ has. By differentiation of (3) and (6) with the value of X_{A} from (15) inserted and using the relations (5) and (8) we find that $\frac{d\,x_{\mathrm{A}}}{d\,k_1} \to 0$ as $k_1 \to \infty$, which means that (22) gives the upper limits for $\gamma_{\mathrm{A\ max.}}$. For γ_{B} an analogous derivation can be made.

Multi-compound systems. The case $k_1 = k_2 = \cdots = k_n = \infty$ implies that all compounds are combined together in one large aggregate. From this and the reasonable simplifying assumption that the number of molecules of A and B is very large we obtain:

$$\left.\begin{aligned} a_{\mathrm{A}} &= x_{\mathrm{A}} = 1;\ a_{\mathrm{B}} = x_{\mathrm{B}} = 0 \\ \gamma_{\mathrm{A}} &= 1/X_{\mathrm{A}};\ \gamma_{\mathrm{B}} = 0 \end{aligned} \quad \bar{n} = \infty \right\}\ \begin{array}{l} \text{valid for} \\ 0 \le X_{\mathrm{B}} \le v/(u+v) \end{array} \qquad (23)$$

$$\left.\begin{aligned} a_{\mathrm{B}} &= x_{\mathrm{B}} = 1;\ a_{\mathrm{A}} = x_{\mathrm{A}} = 0 \\ \gamma_{\mathrm{B}} &= 1/X_{\mathrm{B}};\ \gamma_{\mathrm{A}} = 0 \end{aligned} \quad \bar{n} = \infty \right\}\ \begin{array}{l} \text{valid for} \\ v/(u+v) \le X_{\mathrm{B}} \le 1 \end{array} \qquad (24)$$

We have thus a discontinuity at the point $X_{\mathrm{B}} = v/(u+v)$.

In Figs. 11 and 12, the activity factors and activities have been plotted against X_{B} for $(AB_3)_{n \to \infty}$, $k_1 = k_2 = \cdots = \infty$ (full-drawn curves). From these Figs. it is seen that all of the properties deduced for finite constants are valid also for this case. It is interesting to note the finite values for the maxima of γ_{A} and γ_{B} which can be obtained from (23) and (24):

$$\left.\begin{aligned} \gamma_{\mathrm{A\ max.}} &= (u+v)/u \\ \gamma_{\mathrm{B\ max.}} &= (u+v)/v \end{aligned}\right\} \qquad (25)$$

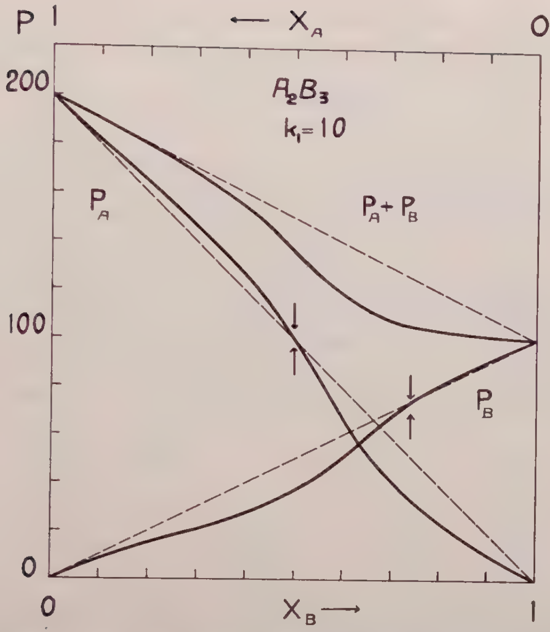


Fig. 13. The vapour pressures P_A, P_B and $P_A + P_B$ are plotted against X_B, assuming the formation of the compound A_2B_3 with $k_1 = 10$ and $P^0_A = 200$ and $P^0_B = 100$. The dashed lines give the same functions if Raoult's law were valid. The arrows at $X_B = 0.5$ and $X_B = 0.75$ indicate the points of intersection of the two types of curves.

The large aggregate, assumed to be formed here, may be considered as a new phase of composition $A_u B_v$. Its solubility in the liquids A and B is negligible and that is why the activities of A and B are either 1 or 0. The discontinuity in the formal activities and activity factors depends upon the fact that the phase A disappears and the phase B appears instead. The system thus illustrates the trivial case of two pure phases in contact with each other.

Heterogeneous equilibria

Activities are often determined by studying the distribution of different components in heterogeneous equilibria. In one phase the activity is known and by determining the distribution the activity in the other phase can be calculated. For instance, activities in liquid binary mixtures are often determined by measuring the partial vapour pressures over the mixture and calculating the activities in the liquid phase using Raoult's law. In order to study how compound formation should influence the distribution, the following two cases will be discussed: vapour-liquid equilibria and ion exchange equilibria.

Vapour-liquid equilibria. In a vapour-liquid equilibrium at moderate pressure, the partial vapour pressures of the components give directly the activities in the liquid phase according to the equations:

$$P_A = P^0_A \cdot a_A = P^0_A \cdot \gamma_A X_A; \quad P_B = P^0_B \cdot a_B = P^0_B \gamma_B X_B. \tag{26}$$

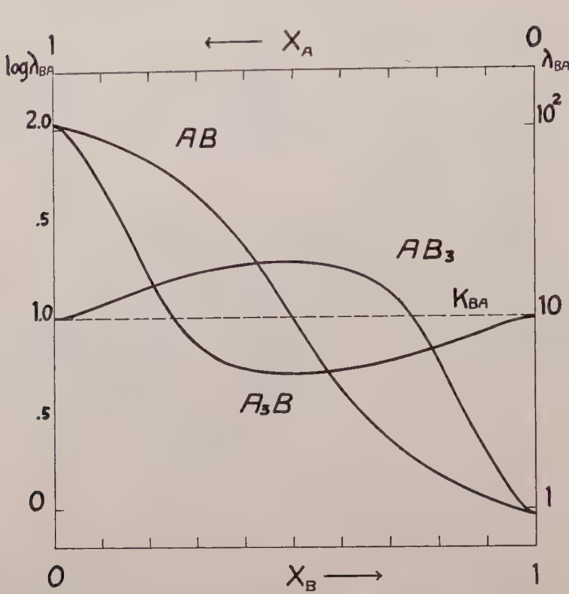


Fig. 14. Log λ_{BA} is plotted against X_B, assuming the formation of one of the following compounds: AB, A_3B and AB_3 with $k_1 = K_{BA} = 10$ and $z_A = z_B = 1$. The value of K_{BA}, which is the average value of λ_{BA}, is indicated by the dashed, straight line.

The intersections of these curves with the straigt lines:

$$P_A = P_A^0 X_A; \quad P_B = P_B^0 X_B \tag{27}$$

are calculated from (11) because the conditions $\gamma_A = 1$ and $\gamma_B = 1$ are fulfilled in the points of intersection of (26) and (27). In Fig. 13, the values of P_A, P_B and $P_A + P_B$ are plotted for A_2B_3, $k_1 = 10$, $P_A^0 = 200$ and $P_B^0 = 100$ (arbitrary units). The arrows indicate the points of intersection at $X_B = 0.5$ and $X_B = 0.75$ which, using (11), give $u = 2$ and $v = 3$. We can thus find the composition of the compound in single-compound systems directly from the vapour pressure curves.

Ion exchange equilibria. Ion exchange equilibria between two exchanging ions have often been treated as two component systems despite the fact that water is also exchanged. It has been shown, HÖGFELDT 1952, that the variation in the activity of water for an ion exchange equilibrium can be neglected if the aqueous solution is dilute. This fact makes the above-mentioned treatment of ion exchange equilibria permissible at least in dilute solutions and the ion exchange reaction between two cations can be written:

$$z_A B^{z_B+} + z_B AR_{z_A} \rightleftharpoons z_B A^{z_A+} + z_A BR_{z_B}, \tag{26}$$

where A^{z_A+}, B^{z_B+} are the two exchanging ions and R^- = the anion framework of the exchanger. For anion exchange equilibria an analogous equation may be written and the results below are equally well applicable to such equilibria.

The equilibrium quotient λ_{BA} of (26) is defined by:

$$\lambda_{BA} = \frac{\{A^{z_A+}\}^{z_B}}{\{B^{z_B+}\}^{z_A}} \frac{X_B^{z_A}}{X_A^{z_B}} = K_{BA} \frac{\gamma_A^{z_B}}{\gamma_B^{z_A}}, \tag{27}$$

where $\{\ \}$ denotes the activity of the component, K_{BA} is the thermodynamic equilibrium constant of (26) and γ_A, γ_B are the activity factors of the components AR_{z_A} and BR_{z_B} in the exchanger, the pure components being taken as the standard states.

In Fig. 14 $\log \lambda_{BA}(X_B)$ has been plotted for some different single-compound systems for $z_A = z_B = 1$, $k_1 = 10$, and $K_{BA} = 10$. For other values of z_A and z_B the $\lambda_{BA}(X_B)$ curves have essentially the same form as those reproduced in Fig. 14. Using equations (13), (27), and that of Gibbs-Duhem, it is easily shown that λ_{BA} has extremes at the points given by (15) independent of the values of z_A and z_B.

From (16) and (27) we find the following limiting values for λ_{BA}:

$$\left.\begin{aligned} X_B = 0;\ \lambda_{BA} = K_{BA} \text{ for } v > 1 \text{ and } \lambda_{BA} = K_{BA}\ (1 + k_1)^{z_A} \text{ for } v = 1 \\ X_B = 1;\ \lambda_{BA} = K_{BA} \text{ for } u > 1 \text{ and } \lambda_{BA} = K_{BA}/(1 + k_1)^{z_B} \text{ for } u = 1 \end{aligned}\right\} \tag{28}$$

From (27) it is easily seen that for $z_A = z_B$ the line $\lambda_{BA} = K_{BA}$ (dotted in Fig. 14) intersects $\lambda_{BA}(X_B)$ at the point $X_B = v/(u + v)$. The shape of $\lambda_{BA}(X_B)$ will thus give valuable information concerning the composition of the compound in a single-compound system and also the value of k_1 if one of u and v is equal to unity.

The methods outlined above for testing the assumption of compound formation are of course also useful for other kinds of heterogeneous equilibria, e.g. partition equilibria etc.

II. Compounds AB_n can be formed up till $n = p$.

For this case we use the same basic assumptions as for (I). We assume the following series of reactions:

$$\left.\begin{aligned}
&\underset{x_A\quad x_B\quad y_1}{A + B \rightleftharpoons AB} && k_1 = \frac{y_1}{x_A \cdot x_B} \\
&\underset{x_B\quad y_1\quad y_2}{B + AB \rightleftharpoons AB_2} && k_2 = \frac{y_2}{x_B \cdot y_1} \\
&\ \vdots\qquad \vdots\qquad \vdots && \ \vdots \\
&\underset{x_B\quad y_{p-1}\quad y_p}{B + AB_{p-1} \rightleftharpoons AB_p} && k_p = \frac{y_p}{x_B \cdot y_{p-1}}
\end{aligned}\right\} \tag{29}$$

Using:

$$q_n = k_1 \cdot k_2 \dots k_n \tag{30}$$

we find:

$$y_n = q_n \cdot x_A \cdot x_B^n. \tag{31}$$

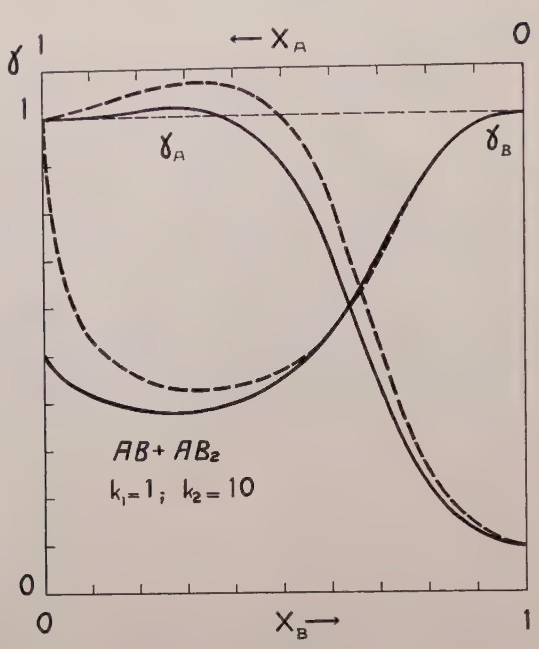


Fig. 15. The activity factors γ_A and γ_B are plotted against X_B, assuming the formation of the following compounds: $AB + AB_2$, $k_1 = 1$ and $k_2 = 10$. For the sake of comparison, the activity factors for the formation of only AB_2, $k_1 = 10$ are dashed. The straight line $\gamma = 1$ is also dashed.

From (3) and (31):

$$x_A + x_B + \Sigma\, q_n\, x_A \cdot x_B^n = 1, \tag{32}$$

where Σ indicates summation over the values $1, 2, \ldots p$.

For X_A and X_B we obtain:

$$\left.\begin{aligned} X_A &= (x_A + \Sigma\, q_n\, x_A \cdot x_B^n)/(x_A + x_B + \Sigma\,(1+n)\, q_n\, x_A \cdot x_B^n) \\ X_B &= (x_B + \Sigma\, n\, q_n\, x_A \cdot x_B^n)/(x_A + x_B + \Sigma\,(1+n)\, q_n\, x_A \cdot x_B^n) \end{aligned}\right\} X_A + X_B = 1. \tag{33}$$

From (8) and (33):

$$\left.\begin{aligned} \gamma_A &= (x_A + x_B + \Sigma\,(1+n)\, q_n\, x_A \cdot x_B^n)/(1 + \Sigma\, q_n\, x_B^n) \\ \gamma_B &= (x_A + x_B + \Sigma\,(1+n)\, q_n\, x_A \cdot x_B^n)/(1 + \Sigma\, n\, q_n\, x_A \cdot x_B^{n-1}) \end{aligned}\right\} \tag{34}$$

and from (12):

$$\bar{n} = \Sigma\, n\, q_n\, x_A \cdot x_B^n / \Sigma\, q_n\, x_A \cdot x_B^n = \Sigma\, n\, q_n\, x_B^n / \Sigma\, q_n\, x_B^n. \tag{35}$$

This number average is the same as that used in complex chemistry. It gives the average number of B bound per molecule AB_n.

Equations (32) ⋯ (35) permit us to calculate γ_A, γ_B and a_A, a_B for different values of p and $k_1, k_2 \ldots k_p$.

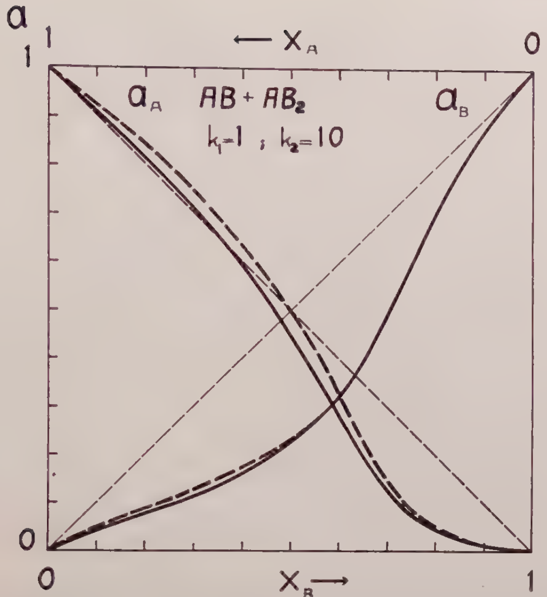


Fig. 16. The activities a_A and a_B are plotted against X_B, assuming the formation of the following compounds: $AB + AB_2$, $k_1 = 1$ and $k_2 = 10$. For the sake of comparison, the activities for the formation of AB_2, $k_1 = 10$ are dashed. The dashed straight lines give a_A and a_B if Raoult's law were valid.

In Figs. 15 and 16, γ_A, γ_B and a_A, a_B are plotted for the system $A + B \rightleftharpoons AB$, $k_1 = 1$; $B + AB \rightleftharpoons AB_2$, $k_2 = 10$. For the sake of comparison the same functions for AB_2, $k_1 = 10$ are dashed. The curves are similar in shape and we shall try to find out if points of intersection etc. can give us any useful information. From equations (32) ... (35) we find:

$$\left.\begin{array}{l} X_B = 0;\ \gamma_A = 1;\ \gamma_B = 1/(1 + k_1);\ \bar{n} = 1 \\ X_B = 1;\ \gamma_B = 1;\ \gamma_A = 1/(1 + \Sigma\, q_n);\ \bar{n} = \Sigma\, n q_n / \Sigma\, q_n > 1 \end{array}\right\} \tag{36}$$

$$\gamma_A = \gamma_B \quad \text{for} \quad X_B = \bar{n}/(1 + \bar{n}) \tag{37}$$

$$\gamma_A = 1;\ x_A = X_A;\ X_B = (\bar{n} - 1)/\bar{n};\ \ \gamma_B = 1;\ x_B = X_B = 1. \tag{38}$$

Of the expressions (36) ... (38) only (36) is of direct value because it permits us to determine the value of k_1 and to obtain some information about the other constants. The relations (37) and (38) are functions of $\bar{n}$ and permit us only to calculate the number average at some different points.

As γ_A intersects the line $\gamma = 1$ at two points, it must have at least one extreme within the interval $0 \leq X_B \leq (\bar{n} - 1)/\bar{n}$.

In Fig. 10, curve 5 gives $\bar{n}(X_B)$ for the system $AB + AB_2$ mentioned above. From this Fig. it is seen that $\bar{n}(X_B)$ increases with X_B in agreement with (36).

It should be mentioned that the relations (36) ... (38) are still valid even if some compounds are missing, i.e. if some of k_n are zero.

For the formation of compounds $A_n B$, analogous expressions will be obtained and are summarized in Table 1 below. From these expressions it is seen that the system $\Sigma A_n B$ is a mirror image of ΣAB_n, which makes it easy to distinguish between the ligand and the central molecule.

Discussion

The influence of u and v. When studying Figs. 1–8 it is seen how very strongly the activity and activity factor curves are influenced by u and v. From these Figs. we find it possible to divide the curves into three main types depending upon their shapes which, in turn, depend upon the values of u and v: 1) Figs. 3, 4, 7 and 8 with $u=v$, 2) Figs. 1 and 5 with u or $v=1$ and 3) Figs. 2 and 6 with $u>1$ and $v>1$, $u \neq v$.

The influence of the equilibrium constants. In Figs. 1, 2, 5, 6, 11 and 12 we can study the influence of the equilibrium constants on the shapes of the activity and activity factor curves. From these Figs. it is seen that the activity factor curves are more sensitive to variations in the equilibrium constants than the activity curves.

It is also seen that when the equilibrium constants increase, the deviations from ideal behaviour increase and the limiting cases shown in Figs. 11 and 12 give the limits for the deviations from the ideal behaviour. The maximum values for γ_A and γ_B given in (22) and (25) represent upper limits as shown above.

The differences between single- and multi-compound systems. By comparing the dashed and full-drawn curves in Figs. 3, 4, 7 and 8, 15 and 16 we can study the possibility of distinguishing between single- and multi-compound systems under the assumption that compound formation is the only reason for the deviations from ideality.

For the full-drawn and dashed activity factor curves in Fig. 3 we find that $u=v$ for both systems. As the full-drawn curves have limiting values different from unity. (16) gives $u=v=1$. Now, if $u=v=1$, it follows from (15) that there should be no extremes in a single-compound system. As the full-drawn curves have extremes we conclude that we must have other compounds as well as AB in the system.

The best relations, however, for distinguishing between single- and multi-compound systems seem to be (11) used on either the activity or the activity factor curves. For instance, by inserting the values of X_B at the points of intersection between the activity curves and the dotted lines in Fig. 7, we find $u=v=2.6$, which shows that we cannot have a single-compound system. When testing real systems it must be kept in mind that other reasons for deviations from ideality also can give u- and v-values different from whole numbers and only a detailed analysis might permit us to distinguish between the multi-compound explanation and other explanations.

When comparing the full-drawn curves in Figs. 3 and 4, we find that the curves are similar in shape; the only difference is the larger deviations from ideality in Fig. 4. The curves might be due to the same mechanism but with different values for the equilibrium constants. That the curves in Fig. 4 correspond to an infinite number of compounds and the curves in Fig. 3 correspond to only two compounds might be determined only by a detailed analysis.

By comparing the dashed and full-drawn curves in Figs. 15 and 16, we find that it is possible to distinguish between single-compound and multi-compound systems here too. The relations (16) and (36) show that for multi-compound systems the limiting values of γ_B for $X_B = 0$ are always different from unity, in contradistinction to the case for the single-compound system. The relations (11) and (38) are also of value for distinguishing between the two kinds of systems. By comparing the relations (10), (11) and (16) with (36) ... (38) we find that it is also possible to distinguish between cases (I) and (II).

Condensed table of relations. In the discussion above a number of the relations previously derived have been used. In the following table all the relations have been summarized, which may be useful when testing binary mixtures for the two special cases (I and II) of compound formation studied above.

Table 1. Some useful relations.

Case No.	$\gamma_A = \gamma_B$ $X_B =$	$\gamma_A = 1$ $X_B =$	$\gamma_B = 1$ $X_B =$	$\gamma_{A\,\max}$, $\gamma_{B\,\max}$ $X_B =$	$X_B = 0$ $\gamma_B =$	$X_B = 1$ $\gamma_A =$
I. ngle-comp. $A_u B_v$	$\frac{v}{u+v}$	$\frac{v-1}{u+v-1}$	$\frac{v}{u+v-1}$	$\frac{1}{u+v}\left[v \pm \sqrt{\frac{u\,v}{u+v-1}}\right]$	$v > 1$ $\gamma_B = 1$ $v = 1$ $\gamma_B = 1/(1+k_1)$	$u > 1$ $\gamma_A = 1$ $u = 1$ $\gamma_A = 1/(1+k_1)$
I. ulti-comp. $(A_u B_v)_n$	$\frac{v}{u+v}$	$\frac{\bar{n}\,v-1}{\bar{n}\,(u+v)-1}$	$\frac{\bar{n}\,v}{\bar{n}\,(u+v)-1}$	–	$v > 1$ $\gamma_B = 1$ $v = 1$ $\gamma_B = 1/(1+k_1)$	$u > 1$ $\gamma_A = 1$ $u = 1$ $\gamma_A = 1/(1+k_1)$
II. $A\,B_n$	$\frac{\bar{n}}{1+\bar{n}}$	$\frac{\bar{n}-1}{\bar{n}}$	1	–	$\gamma_B = 1/(1+k_1)$	$\gamma_A = 1/(1+\Sigma\, q_n)$
II. $A_n\,B$	$\frac{1}{1+\bar{n}}$	0	$\frac{1}{\bar{n}}$	–	$\gamma_B = 1/(1+\Sigma\, q_n)$	$\gamma_A = 1/(1+k_1)$

As $\bar{n}$ in most cases is an unknown function of the composition, the expressions containing $\bar{n}$ do not permit us to calculate u and v from these expressions. They are useful, however, for finding out if the system is a single- or multi-compound one.

When testing a binary mixture using the assumption of compound formation, the data must be in agreement with all of the relations in one of the approaches given in the table, before we can accept this approach as formally applicable to the system in question. One should, however, always bear in mind the possibility that there may be other approaches giving the same types of curves.

Future work. In the literature on binary mixtures there are a number of systems having both positive and negative deviations from Raoult's law, especially among the molten metallic mixtures. It is tempting to investigate such mixtures using the assumption of compound formation, and a following paper will deal with that subject.

A theoretical study of association along the same lines as for compound formation is also in preparation.

Summary

A theoretical study of compound formation in binary mixtures has been undertaken in order to find out how far-reaching are the conclusions concerning the mechanism which can be drawn from the activities and activity factors of the two components.

Of all possible mechanisms for the formation of compounds A_pB_q from the two components A and B the following two cases have been studied:

(I) the compound A_uB_v is formed and either this is the only compound or it associates to larger aggregates $(A_uB_v)_n$, and (II) compounds AB_n are formed by addition of B to A.

A number of simple relations have been derived which permit us to draw conclusions concerning the mechanism from the usual plots of the activities and activity factors against the mole fraction of the components.

These relations permit us, provided only compounds are formed, to distinguish between the cases I and II; to find out if there are more than one compound in the system; to determine the values of u and v in a large number of cases; and, provided u or $v = 1$, to calculate the value of the equilibrium constant for the formation of A_uB_v from the limiting values of the activity factors.

It is also shown how compound formation would influence the shapes of the partial vapour pressure curves in vapour-liquid equilibria and, also, the equilibrium quotient in ion exchange equilibria.

Note: In this paper the activity factors are denoted by γ_A and γ_B in contrast to previous papers (Högfeldt 1951, 1952) in which g_1 and g_2 were used. It is hoped that the advantage of agreement with common practice will outweigh the disagreement with previous papers.

ACKNOWLEDGEMENTS

The author wishes to thank Professor L. G. Sillén, who suggested this work, for many valuable discussions and for his great interest. Thanks are also due to Mr. Erik Ekedahl for valuable discussions and to Mr. D. G. Gillam for correcting the English text.

The Swedish Atomic Energy Committee is thanked for financial support.

Deparment of Inorganic Chemistry, Royal Institute of Technology, Stockholm, Sweden.

REFERENCES

DOLEZALEK, F., 1908. Z. phys. Chem. *64*, 727.
HILDEBRAND, J. H. and EASTMAN, E. D., 1915. J. Am. Chem. Soc. *37*, 2452.
HILDEBRAND, J. H. and SCOTT, R. L., The solubility of nonelectrolytes, Reinhold Publishing Corp. N. Y. 1950.
HÖGFELDT, E., 1951. Acta Chem. Scand. *5*, 1400.
—— 1952. Arkiv Kemi *5*, Nr. 13, 147.
METZGER, G. and SAUERWALD, F., 1950. Z. anorg. Chem. *263*, 324.
REDLICH, O. and KISTER, A. T., 1947. J. Chem. Phys. *15*, 849.
REDLICH, O., 1949. Chem. Revs. *44*, 1.
WASHBURN, E. W., 1912. Trans. Am. Electrochem. Soc. *22*, 330.

Tryckt den 8 juni 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 36

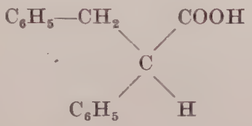
Communicated 24 February 1954 by ARNE FREDGA

Optical resolution of α-phenylhydrocinnamic acid and 2-thenyl-2-thienylacetic acid

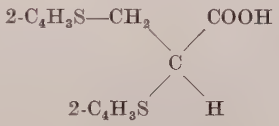
By KURT PETTERSSON

With 4 figures in the text

In connection with work in progress on optically active 2-thiophene-substituted acetic acids (1) it has been desirable to resolve α-phenylhydrocinnamic acid (benzylphenylacetic acid) (I) and 2-thenyl-2-thienylacetic acid (II) into optical antipodes.



(I)



(II)

An attempt to resolve phenylhydrocinnamic acid using strychnine has previously been made by RUPE and KERKOVINS (2). These authors report a melting point of the enanthiomorphs of 83°–89°. This rather large melting-point interval indicates that the resolution was far from complete. After eight crystallisations of the strychnine salt (original weight 24.3 g) the present author obtained only 0.75 g and the liberated acid showed an optical activity of $[\alpha]_D^{25} = -88°$ in benzene as compared with the maximum value of $-134°$. RUPE and KERKOVINS report a maximum optical activity of $[\alpha]_D^{20} = +94.04°$ and $-85.08°$ respectively.

The pure levorotatory α-phenylhydrocinnamic acid is easily obtained by recrystallisation of the *l*-ephedrine salt from acetone. It crystallises from petrol (b.p. 60°–80°) as a colourless powder, melting at 82.5°–84°. Contrary to the optically inactive acid it seems to exist in only one modification. The dextrorotatory acid is obtained from the (−)-α-phenylethylamine salt, purified by recrystallisation from dilute ethanol.

As was earlier pointed out (1) *l*-ephedrine is useful in the resolution of the actual acids. Thus *l*-ephedrine gives the levorotatory 2-thenyl-2-thienylacetic acid in a state of purity by recrystallisation of the salt from benzene. The dextrorotatory acid, however, could not be obtained optically pure in spite of several attempts to isolate it. Quinine, which is the only alkaloid tested giving the (+)-acid in excess, loses its resolving power at a purity of about 70 % of the (+)-acid salt, probably depending upon the formation of mixed crystals. It is of course possible to obtain the acid with *d*-ephedrine, but this substance is not commercially available and the synthesis is rather laborious (3).

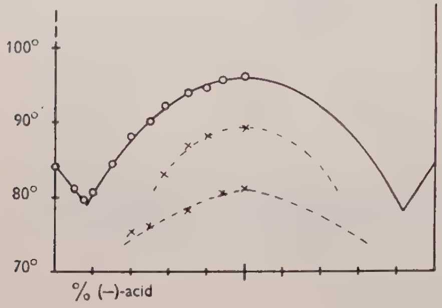


Fig. 1. (+)- and (−)-phenylhydrocinnamic acid.

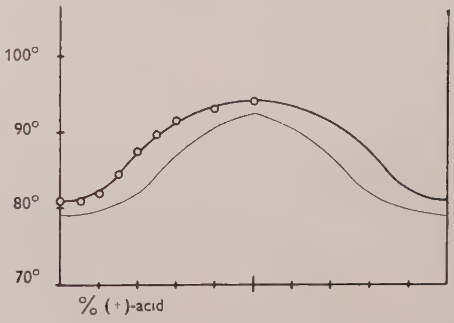


Fig. 2. (+)- and (−)-thenylthienylacetic acid.

The enanthiomorphs of the acids (I) and (II) are thermally stable at their melting point and consequently the melting-point diagrams could be determined in a Kofler hot-stage microscope (4).

It is sometimes possible to determine the steric relation between two compounds by the quasi-racemate method (5, 6). The main factor is that the structural differences between the compounds must not be too great, but also the tendency of the enanthiomorphs to form a true racemate seems to be of some importance. In a study of aryloxy and arylsulphide fatty acids Matell found the formation of quasi-racemates depending more on structural differences than on the tendencies to form true racemates (7) and quasi-racemates could be obtained between compounds with a rather small tendency for true-racemate formation.

Several authors have pointed out that phenyl and 2-thienyl groups fulfil the conditions for isomorphic interchangeability and quasi-racemate formation (8, 9). As is seen from the formulae (I) and (II), 2-thenyl-2-thienylacetic acid (II) may formally be obtained from benzylphenylacetic acid by an exchange of phenyl groups for 2-thienyl groups. The structural differences are then probably greater than between compounds differing by a single such exchange. A similar structural relation exists between 2-thenylphenylacetic acid and benzyl-2-thienylacetic acid. In a previous paper no evidence for a quasi-racemate between these acids could be found (10) and the same would probably be the case for the present acids.

The melting-point diagram of the system (+)- and (−)-benzylphenylacetic acid is shown in Figure 1. It is evident that the enanthiomorphs have a pronounced tendency for racemate formation. The presence of at least three forms of the racemic acid (11) has been verified. Of these the highest melting is evidently a true racemate. Optically inactive 2-thenyl-2-thienylacetic acid is most probably a solid solution of the enanthiomorphs (pseudo-racemate), melting about 13 degrees higher than these (Fig. 2).

The two-component systems (+)-benzylphenylacetic acid — (−)-thenylthienylacetic acid and (−)-benzylphenylacetic acid — (−)-thenylthienylacetic acid show a close resemblance (Figs. 3 and 4). The differences are not pronounced enough to indicate the presence of a quasi-racemate. The thaw-point curves show a far-reaching solubility of thenylthienylacetic acid in benzylphenylacetic acid but

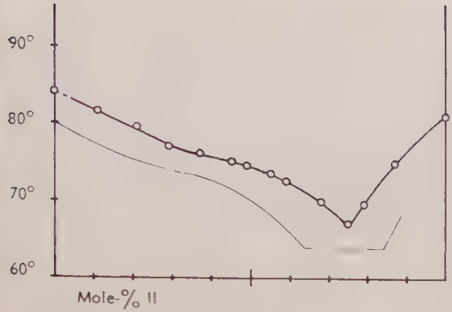


Fig. 3. (+)-Phenylhydrocinnamic acid (I).
(−)-Thenylthienylacetic acid (II).

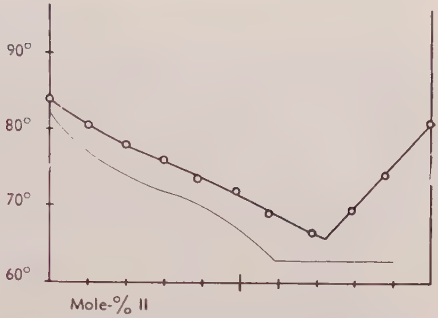


Fig. 4. (−)Phenylhydrocinnamic acid (I).
(−)-Thenylthienylacetic acid (II).

only moderate or slight solubility of benzylphenylacetic acid in thenylthienylacetic acid. This is to be expected, since the unit cell dimensions of phenyl compounds are greater than those of 2-thienyl compounds (8). From considerations of space benzylphenylacetic acid may, at least to a certain extent, be replaced by thenylthienylacetic acid in the lattice of the former, while the reverse exchange in the latter acid requires alterations of the lattice.

X-ray powder photographs have been taken and found to be in accordance with the results of the thermal analysis. The three forms of optically inactive benzylphenylacetic acid showed appreciable differences from the optically active acid and it seems thus probable that they are true racemates. The powder photographs of the optically active and optically inactive thenylthienylacetic acid were very similar and the acids are undoubtedly isomorphic. However, there existed small differences in the photographs which may depend on slightly different positions of the sulphur atoms in the two phases. This would also account for the difference in melting point between the two acids.

The X-ray powder photographs gave no evidence for the existence of a quasi-racemate. The preparations, representing the compositions 50 % in Figs. 3 and 4, gave identical powder photographs, which were also almost identical with the powder photograph of pure (−)-benzylphenylacetic acid. The far-reaching solubility was thus established. It must be remembered, however, that the powder photographs were taken at room temperature and reservations for phase changes at higher temperatures must be made.

Table 1

Solvent	$[\alpha]_D^{25}$ of acid	
	(−)-I	(−)-II
Water (ion)	−236	−153
Ethanol	−277	−196
Acetic acid	−289	−205
Benzene	−303	−193
Acetone	−304	−228

Information about the steric relations between similarly constituted compounds may sometimes be obtained by comparison of the optical activities of the compounds in a number of different solvents. Compounds with the same configuration then show the same mode of variation of the optical activity. The molecular rotations of (−)-α-phenylhydrocinnamic acid, (−)-I, and of (−)-2-thenyl-2-thienylacetic acid, (−)-II, in representative solvents are collected in Table 1. The data constitute a good foundation for the statement that the acids with the same direction of rotation also belong to the same steric series.

Experimental

Optically inactive α-phenylhydrocinnamic acid (I) was prepared from ethyl phenylmalonate and benzyl chloride, followed by hydrolysis and decarboxylation of the resulting ethyl benzylphenylmalonate (12). The crude product was recrystallised repeatedly from petrol (b.p. 60°–80°). The pure acid was obtained as a colourless granular powder, melting at 94°–96°.

Optically inactive 2-thenyl-2-thienylacetic acid was prepared from ethyl 2-thienylcyanoacetate as described in a previous paper (I). After repeated recrystallisations from petrol (b.p. 60°–80°) the acid was obtained as colourless needles or rods melting at 92.5°–94°.

Preliminary tests on resolution: 0.001 moles of the acids I and II and 0.001 moles of the bases were dissolved in 10 ml of hot solvent. The solutions were allowed to stand and in the case of water-soluble solvents water was added gradually until crystals or oils were formed. The crystals were filtered off, the acids liberated and the optical activity measured in absolute ethanol. The results are given in Table 2.

In some cases the salts were too soluble to precipitate even when a large excess of water was added. This is indicated by an *s* in the table.

Table 2.

Base	$[\alpha]_D^{25}$ of acid, measured in ethanol					
	Crystallisation medium					
	Ethanol		Acetone	Benzene	Ethyl acetate	
	I	II	I	II	I	II
Strychnine	−28°	−20°	± 0°			
Quinine	− 6°	+10°	− 6°	+11°		
Brucine	oil		oil	oil		
Quinidine	oil		oil			
Cinchonidine	−10°	− 7°	− 23°			
Cinchonine	oil	s	oil			
Morphine	oil	oil	oil	s		
(−)-Phenethylamine	+12°	± 0°				
l-Ephedrine	−56°	s	−115°	−68°	−19°	
Nicotine		s	s			
(+)-Benzedrine	+ 5°	± 0°		± 0°		−3°
Yohimbine		oil				

Optical resolution of phenylhydrocinnamic acid: 9.5 g (0.04 moles) of optically inactive phenylhydrocinnamic acid and 9.0 g of *l*-ephedrine dihydrate were dissolved in 150 ml of hot acetone. The solution was allowed to cool and crystallise for 12 hours in a refrigerator. The precipitate was filtered off, dried and weighed. About 0.5 g was set apart, the acid was liberated and the optical activity measured in acetone. The remaining part of the precipitate was recrystallised from acetone, until the optical activity of the liberated acid remained constant. The precipitate is rather difficult to dissolve in the appropiate amounts of the solvent, and it is necessary to reflux for a few minutes. The course of the resolution was as follows:

Crystallisation No.	1	2	3	4	5
ml of acetone	150	50	22	12	8
g of salt obtained	4.6	2.0	1.2	0.8	0.5
$[\alpha]_D^{25}$ of acid (acetone)	$-81°$	$-116°$	$-122°$	$-132°$	$-132°$

The mother liquor from the first crystallisation was evaporated to dryness at room temperature and the residue recrystallised from the successive mother liquors in the first series of crystallisations. In this way a further 2.0 g of salt was obtained. The liberated acid showed an optical activity of $-132°$. The total yield of salt was thus 2.5 g.

The mother liquor from the first crystallisation of the second series was evaporated to dryness at room temperature and the acid was liberated from the residue. 2.5 g (0.011 moles) of the acid ($[\alpha]_D^{25} = +75°$ in ethanol) and 1.4 g (0.011 moles) of (−)-α-phenylethylamine were dissolved in a warm mixture of 13 ml of ethanol, 13 ml of acetone and 26 ml of water. The salt obtained was recrystallised until the optical activity of the acid (measured in ethanol) remained constant.

Crystallisation No.	1	2	3	4
ml of ethanol	13	13	10	10
ml of acetone	13	20	16	16
ml of water	26	23	18	18
g of salt obtained	3.6	2.8	2.4	1.9
$[\alpha]_D^{25}$ of acid (ethanol)	$+89°$	$+114°$	$+122°$	$+122°$

(−)-α-Phenylhydrocinnamic acid: 2.5 g of the *l*-ephedrine salt were decomposed with 2-*N* sulphuric acid and the liberated acid was extracted with ether. After removal of the ether by distillation 1.4 g of crude acid remained. It was recrystallised from petrol (b.p. 60°–80°) from which it was obtained as a colourless powder, melting at 82.5°–84°.

47.59 mg of acid, 4.68 ml of 0.04475-C NaOH
$C_{15}H_{14}O_2$. Equiv. wt. calc. 226.1, found 227.2.

Weighed amounts of acid were made up to 10.01 ml with different solvents and the optical activity measured. In the case of water the acid was dissolved in 2.0 ml of ethanol, neutralised with 0.1-C NaOH and finally made up to 10.01 ml with water.

Solvent	mg of acid	$2\alpha_D^{25}$	$[\alpha]_D^{25}$	$[M]_D^{25}$
Acetone	49.1	$-1.318°$	$-134.3°$	$-303.7°$
Ethanol	51.3	$-1.258°$	$-122.7°$	$-277.4°$
Benzene	50.5	$-1.350°$	$-133.8°$	$-302.5°$
Glacial acetic acid	62.2	$-1.582°$	$-127.3°$	$-287.8°$
Water (ion)	51.1	$-1.065°$	$-104.3°$	$-235.8°$

(+)-*α-Phenylhydrocinnamic acid:* 1.9 g of the (−)-α-phenylethylamine salt were decomposed with 2-*N* sulphuric acid and the liberated acid was extracted with ether and purified as described for its optical antipode, which it resembles in all respects. M.p. 82.5°–84°.

60.76 mg of acid, 6.00 ml of 0.04475-C NaOH

$C_{15}H_{14}O_2$. Equiv. wt. calc. 226.1, found 226.4

55.4 mg of the acid in 10.01 ml of acetone: $2\alpha_D^{25} = +1.483°$, $[\alpha]_D^{25} = +133.8°$, $[M]_D^{25} = +302.5°$.

Resolution of 2-thenyl-2-thienylacetic acid: 7.5 g (0.031 moles) of optically inactive acid and 5.8 g of *l*-ephedrine dihydrate were dissolved in 200 ml of hot benzene. The solution was allowed to crystallise at room temperature for 12 hours. The precipitate was filtered off, dried and weighed. About 0.5 g was set aside, the acid liberated and the optical activity measured in acetone. The principal part of the salt was recrystallised from benzene and the procedure repeated until the optical activity of the acid remained constant.

Crystallisation No.	1	2	3	4
ml of benzene	200	50	30	30
g of salt obtained	5.1	3.6	3.3	3.1
$[\alpha]_D^{25}$ of acid (acetone)	$-82°$	$-88°$	$-92°$	$-92°$

The only alkaloid tried, giving (+)-thenylthienylacetic salt in excess is quinine. This alkaloid loses its resolving power at an optical activity of the acid of about +70°. Thus the specific rotation of the acid was raised from +65° to +71° by five crystallisations from dilute acetone.

Attempts to concentrate the (+)-acid salt in the above-mentioned series by partial evaporation of the solvent were without success.

Attempts to concentrate the (+)-acid by recrystallisation of a moderately dextrorotatory acid from petrol (b.p. 60°–80°) in the usual way and according to a filter-paper method (13) were unsuccessful.

(−)-*2-Thenyl-2-thienylacetic acid:* 3.1 g of the *l*-ephedrine compound were decomposed with 2-*N* sulphuric acid and the liberated acid was extracted with ether. The ether was removed and the crude acid (1.7 g) was recrystallised from petrol (b.p. 60°–80°). 1.4 g of acid, melting at 79°–81° was obtained as colourless needles or rods.

81.35 mg of acid, 7.64 ml of 0.04475-C NaOH

$C_{11}H_{10}O_2S_2$. Equiv. wt. calc. 238.1, found 237.9.

Weighed amounts of the acid were made up to 10.01 ml with different solvents and the optical activity measured. In the case of water the acid was dissolved in 2.0 ml of ethanol, neutralised with 0.1-C NaOH and finally made up to 10.01 ml with water.

Solvent	mg of acid	$2\alpha_D^{25}$	$[\alpha]_D^{25}$	$[M]_D^{25}$
Acetone	126.9	− 2.434°	− 95.9°	− 228.4°
Ethanol	95.3	− 1.570°	− 82.4°	− 196.2°
Benzene	93.7	− 1.520°	− 81.2°	− 193.2°
Glacial acetic acid	98.1	− 1.688°	− 86.1°	− 204.6°
Water (ion)	89.5	− 1.152°	− 64.4°	− 153.4°

Table 3. (+)- and (−)-Phenylhydrocinnamic acid.

% (−)-acid	Melting point		
0	84°		
5	81°		
7.5	79.5°		
10	80.5°		
15	84.5°		
20	88°		75°
25	90°		76°
29	92°	83°	
35	94°	87°	78°
40	94.5°	88°	
44	95.5°		80.5°
50	96°	89°	81°

Table 4. (+)- and (−)-Thenylthienylacetic acid.

% (+)-acid	M. p.
0	79° − 81°
5	79° − 81°
10	79° − 82°
15	80° − 84.5°
20	82° − 87.5°
25	84° − 89.5°
30	86° − 91.5°
40	90.5° − 93°
50	92.5° − 94°

Table 5. (+)-Phenylhydrocinnamic acid (I). (−)-Thenylthienylacetic acid (II).

Mole-% II	M. p.
0	82.5° − 84°
11	77° − 81.5°
21	75° − 79.5°
29	74° − 77°
37	73° − 76°
45	72° − 75°
49	70° − 74.5°
55	67° − 73.5°
59	66° − 72.5°
68	64° − 70°
75	64° − 67°
79	64° − 69.5°
87	67° − 75°
100	79° − 81°

Table 6. (−)-Phenylhydrocinnamic acid (I). (−)-Thenylthienylacetic acid (II).

Mole-% II	M. p.
0	82.5° − 84°
10	77° − 80.5°
19.5	74° − 78°
30	72° − 76°
39	70° − 73.5°
49	67° − 72°
57.5	63.5° − 69°
69	63° − 66.5°
79	62.5° − 69.5°
88	63° − 74°
100	79° − 81°

Melting-point diagrams

Thermal stability of the enanthiomorphs: Optically active acids were heated above their melting points (90°–100°) for about half an hour. The optical activities were measured in acetone before and after heating.

(−)-Phenylhydrocinnamic acid:

Before heating: 102.2 mg in 10.01 ml 2 $\alpha_D^{25} = -1.43^\circ$, $[\alpha]_D^{25} = -70^\circ$
After heating: 91.1 mg in 10.01 ml 2 $\alpha_D^{25} = -1.27^\circ$, $[\alpha]_D^{25} = -70^\circ$

(−)-Thenylphenylacetic acid:

Before heating: 156.0 mg in 10.01 ml 2 $\alpha_D^{25} = -2.75^\circ$, $[\alpha]_D^{25} = -88^\circ$
After heating: 132.0 mg in 10.01 ml 2 $\alpha_D^{25} = -2.36^\circ$, $[\alpha]_D^{25} = -89^\circ$

Melting-point preparations: Weighed amounts of the components (total weight 10 mg) were dissolved in a few drops of acetone ("AnalaR" standards for laboratory chemicals). The solvent was evaporated at room temperature and the residues were carefully powdered and dried in a desiccator. The melting points were determined in a hot-stage microscope (4).

X-ray powder photographs: The melting-point preparations to be investigated were exposed for 20 minutes in Cu-K radiation (45 kV, 25 mA), using a camera of GUINIER type and an aluminium single crystal with double curvature as monochromator (14).

Summary

α-Phenylhydrocinnamic acid has been resolved into optical antipodes. The optical rotation of the enanthiomorphs far exceed earlier reported values. The existence of three forms of optically inactive acid has been verified.

(−)-2-Thenyl-2-thienylacetic acid has been isolated from the optically inactive acid by means of *l*-ephedrine. The optically inactive 2-thenyl-2-thienylacetic acid is most probably a pseudo-racemate i.e. a solid solution of the enanthiomorphs.

The four possible two-component systems of the four enanthiomorphs have been investigated in a hot-stage microscope and the results discussed.

The author is indebted to Professor ARNE FREDGA for valuable discussions in connection with the present work. Part of the expenses has been defrayed by a grant from the Nobel fund of the Royal Swedish Academy of Science (Kemiska Prisgruppens särskilda fond).

Organic Department, Institute of Chemistry, University of Uppsala, February 1954.

REFERENCES

1. FREDGA, A., and PETTERSSON, K., Acta Chem. Scand. *4*, 1306 (1950).
2. RUPE, H., and KERKOVINS. W., Ber. *45*, 1398 (1912).
3. NAGAI, W. N., and KANAO, S., Ann. *470*, 157 (1929).
4. KOFLER, L. and A., Mikromethoden zur Kennzeichnung organischer Stoffe und Stoffgemische. Innsbruck 1948.
5. FREDGA, A., "The Svedberg 1884 30/8 1944". Uppsala 1944, p. 261.
6. MATELL, M., Mikrochem. ver. Microchim. Acta *38*, 532 (1951).
7. MATELL, M.. Stereochemical Studies on Plant Growth Substances. Diss. Uppsala 1953 p. 25.
8. GRONOWITZ, S., Arkiv Kemi *6*, 284 (1953).
9. FREDGA, A., and PALM, O., Arkiv Kemi, Mineral. Geol. *26A*, nr 26 (1949).
10. PETTERSSON, K., This journal. In the press.
11. v. MILLER, W., and ROHDE, G., Ber. 25, 2018 (1892).
12. WISLICENUS, W., and GOLDSTEIN, K., Ber. *28*, 818 (1895).
13. VASISTH, R. C., and MUTHANA, M. S., Nature *172*, 862 (1953).
14. HÄGG, G., and KARLSSON, N., Acta Cryst. *5*, 728 (1952).

Tryckt den 8 juni 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 37

Communicated 10 March 1954 by ARNE FREDGA

Phase diagrams of phenylsuccinic and 2-thienylsuccinic acids. Configuration of phenylsuccinic acid.

By KURT PETTERSSON

With 4 figures in the text

It has been shown by FREDGA (1) that aromatic and aliphatic compounds may be related sterically by means of ring fission of thiophene compounds. In this way 2-thenylsuccinic acid was converted to the sterically related *n*-pentylsuccinic acid. The present author has in the same way transformed 2-thienylsuccinic acid to *n*-butylsuccinic acid, through which the configuration of the former acid was determined (2). If the configuration of 2-thienylsuccinic acid and phenylsuccinic acid can be related in some way, the configuration of the latter acid relative to the glyceraldehyde is determined.

Work in progress at this laboratory indicates that the phenyl and thienyl groups in many cases fulfil the conditions of isomorphic exchangeability and quasi-racemate formation (3–4). It was possible, therefore, that phenylsuccinic acid and 2-thienylsuccinic acid could be related by the quasi-racemate method (5–6). A thermal analysis of the phase diagrams must be avoided, however, since it was shown (2) that 2-thienylsuccinic acid decomposes too rapidly at its melting point. Hence the systems were investigated with X-rays.

The optical resolution of phenylsuccinic acid has been carried out by WREN and WILLIAMS (7). Some of their results indicate that the enanthiomorphs decompose at the melting point (anhydride formation and racemisation). FREDGA and MATELL have investigated the two-component system (+)- and (–)-phenylsuccinic acid in a hot-stage microscope (8), using a method of instantaneous melting described by TIMMERMANS (9). According to these authors the enanthiomorphs and racemate of the acid are dimorphic, which in the case of the racemate was also reported by SCHAUM, SCHAELING and KLAUSING (10). FREDGA and MATELL suggest that the stable modification of the optically inactive acid is a solid solution of the unstable modification of the enanthiomorphs (pseudo-racemate) and that the unstable modification of the optically inactive acid is a conglomerate of the stable modifications of the enanthiomorphs.

The systems have now been investigated with X-ray at room temperature. The existence of polymorphic modifications of the enanthiomorphs has been verified. An authentic sample of (–)-phenylsuccinic acid, prepared byFREDGA and MATELL (8) and recrystallised from water, gives the powder photograph shown in Fig. 1 a. If an ether solution of the acid is allowed to evaporate slowly, the acid is obtained partly as a granular powder and partly as rods. The rods can be used

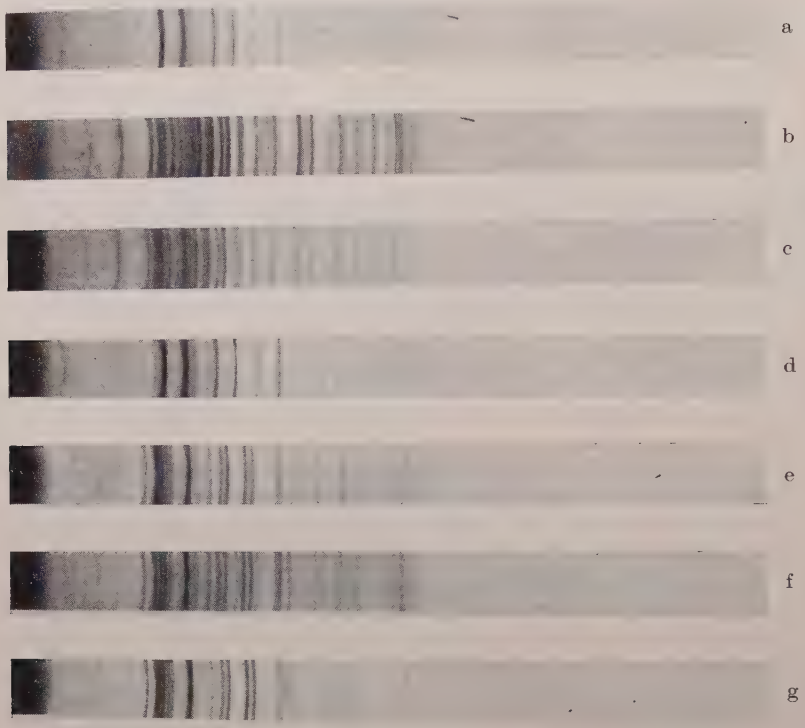


Fig. 1. (+)- and (–)-Phenylsuccinic acid. a. "High temperature" (–)-phenylsuccinc acid. b. "Low temperature" (–)-phenylsuccinic acid. c. 10% (+)-phenylsuccinic acid. "Low temperature" form. d. 10% (+)-phenylsuccinic acid. "High temperature" form. e. 41% (+)-phenylsuccinic acid. "Low temperature" form. f. 41% (+)-phenylsuccinic acid. "High temperature" form. g. Racemic phenylsuccinic acid.

to seed a water solution of the acid, which then crystallises as fine needles. The powder photograph of these (Fig. 1 b) is quite different from that of the original acid. The needles have been stored for three months without visible change, but when heated they convert to the first-mentioned modification and thus represent a "low temperature" form of the optically active phenylsuccinic acid. This modification is probably not the same as the unstable modification obtained by Fredga and Matell and it seems not unlikely that the enanthiomorphs are trimorphic.

It is interesting to note that the "low temperature" modification of the optically active phenylsuccinic acid its obtained on seeding a water solution with the "low temperature" enanthiomorph of 2-thienylsuccinic acid having the same direction of rotation (2). Hence the two modifications seem to be isomorphic, which has also been verified with X-rays (Fig 2, a and b). The similarities between the two powder photographs are obvious, almost the only difference being the spacing of the lines. This indicates that the unit cell dimensions are somewhat greater in phenylsuccinic acid. Gronowitz (4) found the same similarities between benzylsuccinic acid and the isomeric thenylsuccinic acids.

The powder photograph of the inactive phenylsuccinic acid, prepared by Fredga and Matell (8), differs from those obtained from the optically active acids

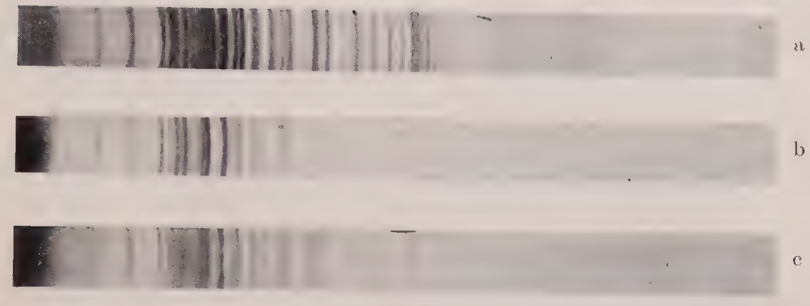


Fig. 2. Isomorphy between optically active phenylsuccinic and 2-thienylsuccinic acids. a. "Low temperature" phenylsuccinic acid. b. "Low temperature" 2-thienylsuccinic acid. c. Equimolecular parts of (+)-phenylsuccinic acid and (+)-2-thienylsuccinic acid.

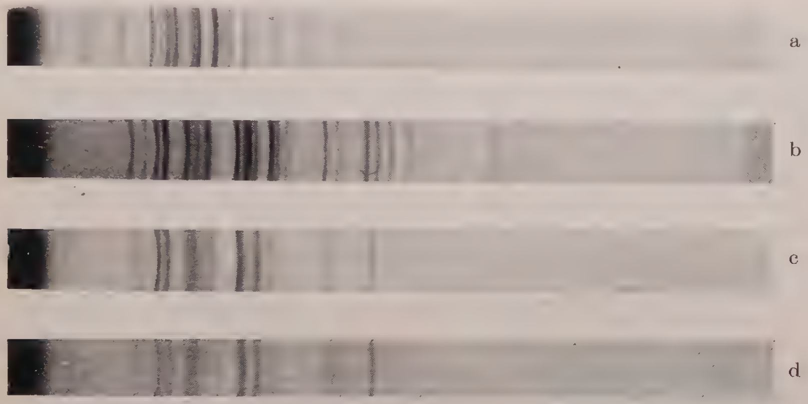


Fig. 3. (+)- and (–)-2-Thienylsuccinic acid. a. "Low temperature" (+)-2-thienylsuccinic acid. b. "High temperature" (+)-2-thienylsuccinic acid. c. 10% (–)-2-thienylsuccinic acid. d. Inactive 2-thienylsuccinic acid.

(Fig. 1, a, b and g). Hence it is not a pseudo-racemate of these modifications. The behaviour of the analogously constituted 2-thienylsuccinic acid (*vide infra*) makes it possible, however, that the inactive acid may be a solid solution of a third modification of the enanthiomorphs.

Several attempts to prepare the unstable modification of the optically inactive phenylsuccinic acid for X-ray analysis have been unsuccessful.

In the concentration range between 0% and 50% of one of the enanthiomorphs two points have been investigated (about 10% and 41% of (+)-phenylsuccinic acid). The samples were dissolved in acetone, the solvent removed without heating and the residue exposed to X-rays at room temperature. In this condition the samples are composed of a mixture of inactive acid and the "low temperature" modification of the enanthiomorph (Fig. 1, c and e). After heating to 140° followed by rapid cooling to room temperature the preparations were composed of optically inactive acid and the "high temperature" modification of the enanthiomorph (Fig. 1, d and f). The transformation temperatures have not been determined.

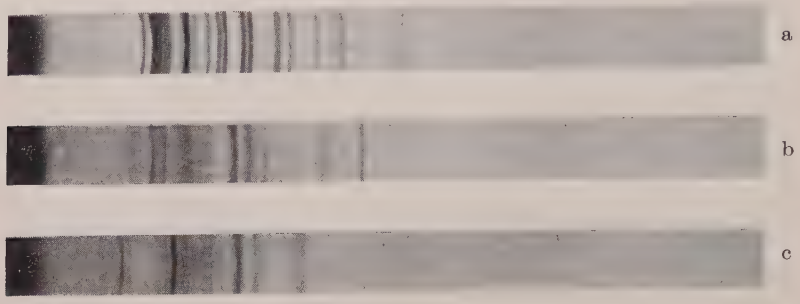


Fig. 4. Inactive acids and quasi-racemate. a. Phenylsuccinic acid. b. 2-Thienylsuccinic acid. c. Equimolecular amounts of (–)-phenylsuccinic acid and (+)-2-thienylsuccinic acid (quasi-racemate).

Optically active 2-thienylsuccinic acid crystallises from water as rods or as a granular powder, both of which show the same powder photograph (Fig 3 a). This represents a "low temperature" modification, for at higher temperatures a transformation to a "high temperature" modification (Fig. 3 b) occurs without racemisation (2).

It is interesting to note that in the concentration range between 0.8% and 99.2% of one of the enanthiomorphs the same phase exists both at low and high temperatures (Fig. 3, c and d) (the points 0.8; 2; 5; 10; 40 and 50% have been investigated). This phase differs distinctly from the two obtained of the enanthiomorphs although some similarities to the "high temperature" modification of the enanthiomorphs are present.

The behavoir between 0% and 0.8% is not easy to find out with X-rays. It seems rather probable that the enanthiomorphs are completely soluble in each other, the homogeneous phase extending through the whole diagram. The enanthiomorphs must then be trimorphic. On the other hand, the existence of a eutectic must not be fully excluded. An attempt to prepare a third modification of (+)-2-thienylsuccinic acid by seeding a solution at room temperature with a small amount of the 0.8% preparation gave no definite information of the structure, since the product obtained was a mixture of the "low temperature" modification with either the "high temperature" modification or with a third modification, isomorphous to the inactive acid. Since the "high temperature" modification has never before been obtained at room temperature, the latter suggestion may be more probable.

A mixture of equivalent parts of (+)-2-thienylsuccinic acid and (–)-phenylsuccinic acid gives a powder photograph quite different from all of the others (Fig. 4 c). Since a mixture of equivalent parts of (+)-2-thienylsuccinic acid and (+)-phenylsuccinic acid forms a solid solution of the "low temperature" modifications of the components (Fig. 2), the formation of a compound between the enanthiomorphs with opposite direction of rotation must be due to steric causes. Hence this compound must be classified as a quasi-racemate and hence its components have opposite configurations.

As is known, the molecular rotation of similary constituted compounds with the same configuration varies in the same manner when measured in a number of representative solvents. The data for the dextrorotatory phenylsuccinic and 2-thienylsuccinic acids are given in Table 1 and constitute a good foundation for the statement that the two acids have the same configuration.

Table 1.

Solvent	Molecular rotation $[M]_D^{25}$	
	(+)-phenylsuccinic acid	(+)-2-thienylsuccinic acid
Water (ion)	+148°	+ 81.8°
Ethanol	+280°	+206°
Acetic acid	+317°	+226°
Acetone	+333°	+253°
Ethyl acetate	+336°	+253°

From the formation of a quasi-racemate and from the behaviour of the molecular rotations in different solvents it is evident that (+)-phenylsuccinic acid and (+)-2-thienylsuccinic acid belong to the same steric series. As was shown in a previous paper (2), (+)-2-thienylsuccinic acid belongs to the D-glyceraldehyde system and consequently the same must be valid for (+)-phenylsuccinic acid and related substances.

Experimental

Attempts to prepare the instable modification of racemic phenylsuccinic acid:
A sample of racemic phenylsuccinic acid was

(*a*) melted and allowed to crystallise rapidly

(*b*) melted and just before melting began seeded with "high temperature" (−)-phenylsuccinic acid

(*c*) heated for two hours at 148°–155° in the presence of a small amount of "high temperature" (−)-phenylsuccinic acid

(*d*) synthesised by dissolving equal amounts of the "high temperature" enanthiomorphs in a small amount of acetone ("AnalaR" reagent for laboratory chemicals), followed by removal of the solvent at room temperature in the presence of small amount of undissolved enanthiomorph.

The four preparations were investigated with X-rays. The powder photographs were identical with each other and with that of the ordinary racemic acid.

The heating of the samples was carried out in stoppered test tubes, contained in a larger test tube, which was fitted with a thermometer and heated in an oil bath at about 140° for two hours.

X-ray powder photographs were taken in a focusing camera of Guinier type with CuK radiation (45 kV, 25 mA), using an aluminium single crystal with double curvature as monochromator (11). Because of the high intensity and consequently low time of exposure (twenty minutes) this monochromator is very useful in the kind of work described in this paper.

Racemisation of optically active phenylsuccinic acid: Two samles of optically active phenylsuccinic acid ($[\alpha]_D^{25} = -171^\circ$) were heated for two hours at 140°–145° and 158°–160° respectively. The first sample showed $[\alpha]_D^{25} = -171^\circ$. The second sample was fluid at the end of the experiment and was almost completely racemised.

A sample of (+)-phenylsuccinic acid was melted at as low temperature as possible for one minute and then allowed to cool rapidly. $[\alpha]_D^{25} = +123^\circ$. All activities were measured in acetone.

Optical activities in different solvents: Weighed amounts of the acids were dissolved in 10.01 ml of solvent and the optical activity measured. In the case of water the acids were neutralised with 0.1-C NaOH and made up to 10.01 ml with water.

The data are given in the Tables 2 and 3.

Table 2. Optical activity of phenylsuccinic acid.

Solvent	mg of acid	$2\alpha_D^{25}$	$[\alpha]_D^{25}$	$[M]_D^{25}$
Water (ion)	74.2	+1.129°	+ 76.1°	+147.7°
Ethanol	86.1	+2.480°	+144.1°	+279.7°
Acetic acid	96.3	+3.148°	+163.5°	+317.4°
Acetone	94.0	+3.220°	+171.4°	+332.7°
Ethyl acetate	80.0	+2.767°	+173.0°	+335.8°

Table 3. Optical activity of 2-thienylsuccinic acid.

Solvent	mg of acid	$2\alpha_D^{25}$	$[\alpha]_D^{25}$	$[M]_D^{25}$
Water (ion)	65.4	+0.535°	+ 40.9°	+ 81.8°
Ethanol	83.7	+1.720°	+102.8°	+205.7°
Acetic acid	82.1	+1.850°	+112.8°	+225.7°
Aceton	78.7	+1.960°	+124.6°	+249.2°
Ethyl acetate	82.1	+2.070°	+126.2°	+252.5°

SUMMARY

The phase diagrams of phenylsuccinic acid and 2-thienylsuccinic acid have been investigated with X-rays. The enanthiomorphs of the acids have been found to be polymorphic, those of phenylsuccinic acid at least dimorphic and those of 2-thienylsuccinic acid probably trimorphic. Optically inactive phenylsuccinic acid has been found to act as an ordinary true racemate, whereas optically inactive 2-thienylsuccinic acid probably is a solid solution of the enanthiomorphs (pseudo-racemate).

The optically active phenylsuccinic acid and 2-thienylsuccinic acid with opposite direction of rotation have been found to give a quasi-racemate and therefore have opposite configurations. The acids with the same direction of rotation form a solid solution. Since (+)-2-thienylsuccinic acid belongs to the D-glyceraldehyde system, the same is valid for (+)-phenylsuccinic acid and related substances.

The optical activities of phenylsuccinic acid and 2-thienylsuccinic acid have been measured in different solvents and the data compared.

ACKNOWLEDGEMENTS

Professor ARNE FREDGA and Dr. MAGNUS MATELL kindly placed the enanthiomorphs and the racemate of phenylsuccinic acid at my disposal. I take this opportunity of expressing my sincere gratitude for this and for valuable discussions in connection with the present paper.

I also wish to express my thanks to the head of this institute, Professor GUNNAR HÄGG, for his kindness in placing the X-ray apparatus at my disposal, and to Dr. NILS SCHÖNBERG (KARLSSON) for his expert advice in connection with the X-ray photographs.

Department of Organic Chemistry, Chemical Institute, University of Uppsala, March 1954.

REFERENCES

1. FREDGA, A., Arkiv Kemi *6*, 277 (1953).
2. PETTERSSON, K., Arkiv Kemi *7*, 39 (1954).
3. FREDGA, A., and PALM, O., Arkiv Kemi, Mineral. Geol. *26A* nr 26 (1949).
4. GRONOWITZ, S., Arkiv Kemi *6*, 283 (1953).
5. FREDGA, A., "The Svedberg 1884 30/8 1944", Uppsala 1944, p. 261.
6. MATELL, M., Mikrochemie ver. Microchim. Acta *38*, 532 (1951).
7. WREN, H., and WILLIAMS, H., J. Chem. Soc. *109*, 572 (1916).
8. FREDGA, A., and MATELL, M., Bull. Soc. Chim. Belge *62*, 47 (1953).
9. TIMMERMANS, J., Bull. Soc. Chim. Belge *40*, 105 (1931).
10. SCHAUM, K., SCHAELING, K., and KLAUSING, F., Ann. *411*, 193 (1916).
11. HÄGG, G., and KARLSSON, N., Acta Cryst. *5*, 728 (1952).

Tryckt den 8 juni 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 38

Communicated 10 March 1954 by H. von Euler and Karl Myrbäck

Studies on the spleen from rats fed with N,N-dimethyl-4-aminoazobenzene

By Stig Åkerfeldt

With 1 figure in the text

It is known that the spleen in rats experiences certain changes when the animals are fed with the cancerogen N,N-dimethyl-4-aminozobenzene, BY (Butter Yellow). Lowenhaupt (1) reports the occurrence of lymphoblastic lymphosarcomas in some of the spleens from a number of BY-fed rats. Edwards and White (2) found an increase in weight and a change in colour of this organ after BY feeding. These latter observations were later confirmed by Euler (3). So far, however, no further examination of these changes of the spleen has been carried out.

In the present investigation special attention has been paid to different biochemical changes in the spleen of BY-fed rats. Especially the content of hemoglobine and free amino acids of this organ proved to be interesting subjects for examination. In connection with these studies it was found probable that the weight increase of the spleen, which takes place when rats are fed with BY, is intimately associated with the other two subjects under examination and, consequently, this question was also studied.

In this investigation rats of the white Wistar strain were used exclusively. These rats were fed with a diet, the composition of which has been published previously (37). By the time of development of the hepatoma (4–8 months) the animals had orally received 1–2 g of BY.

Results

Increase in weight of the spleen

According to our experiments the weight increase of the spleen starts as soon as BY is supplied to the animal (Fig. 1). The spleen weight reaches a maximum at about the time for, or a little after, the development of the hepatoma, whereafter it decreases. Finally, after about 15–20 months of BY feeding, the spleen approaches its normal weight, provided that the animals survive.

Both the liver and the total body of the Wistar rats were found to change in weight during the administration of BY. For normal rats a surprisingly constant value of the ratio liver weight/spleen weight was found (Table 1). This ratio is less constant in rats fed with BY, but the same mean value of the ratio is obtained.

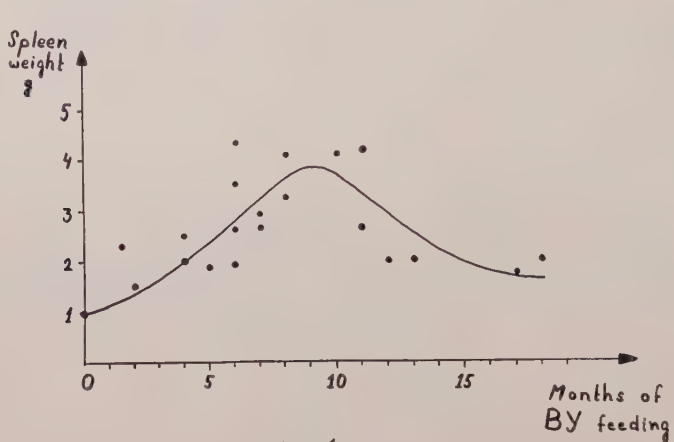


Fig 1.

Table 1. Values from 20 controls and 20 BY-fed rats. For the last species the mean values are given after BY feeding during 1–15 months.

Animal	Total body weight (g)	Liver weight (g)	Spleen weight (g)	Liver/Spleen
Normal	320±40	11± 2	1.0±0.1	11
BY-fed	246±40	28±10	2.6±1.6	11

Change in colour of the spleen

In all the BY-fed rats examined the spleens (BY-spleens) were found much darker in colour than normal spleens. It appeared that this fact is due partly to an abnormally high hemoglobine content of these spleens (Table 2). The hemoglobine determinations were carried out according to GREENBERG and ERICKSON (5).

Table 2. Hemoglobine content in mg per g fresh tissue.

Liver		Spleen		BY administration, months
Normal	BY	Normal	BY	
6.1	14.8	40.5	103	4
9.8	12.7	14.6	53.3	7
10.4	9.0	23.2	56.6	8
19.8	7.7	34.3	58.1	8
11.1	11.8	31.4	58.8	8
mean 11	11	29	66	

Absorption spectra of hemoglobine from BY-spleens and normal spleens were found to be identical. The blood of our BY-fed Wistar rats showed the normal—or sometimes a slightly low—hemoglobine content. In all kinds of cancer, however, a low value of the blood hemoglobine content is common (6, 7, 8) and has been reported also for BY-fed rats (6). Another blood constituent, the water soluble c-hemine of SCHALES (9), was found to decrease to 65–70 % of the normal value in our BY-fed rats. A considerable part of the lacking c-hemine was found in the BY-spleen, where it contributes to the abnormally deep colour of this organ.

Quantitative determination of the free amino acids of the spleen

In the blood of rats with Jensen sarcoma a decrease in the amount of free histidine and arginine has been reported (10, 11). This fact actualized a quantitative determination of the free amino acids in the spleen of our BY-fed rats. Using microbiological methods SOLOMON *et al.* (12) quantitavely estimated 12 free amino acids in normal rat spleens. The present paper chromatographic investigation has shown the presence of considerably more free amino acids in this organ. Using an elution technique by AWAPARA (13) and a spot area method for quantitative determination of amino acids on two dimensional paper chromatograms by ÅKERFELDT (14), the amounts of free amino acids were estimated (Table 3). Furthermore small amounts of proline and oxiproline were found.

Preliminary experiments have shown that generally a low value of the free amino acid content is found also in the blood of our BY-fed rats.

Table 3. Mean values of 3 normal and 3 BY-fed rats, which had received BY during 6 months.

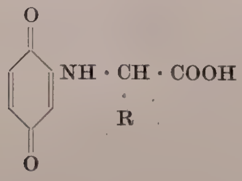
Amino acid	Normal spleen mg/100 g fresh tissue	BY-spleen mg/100 g fresh tissue
Alanine	21 ± 4	11 ± 2
γ-Amino butyric acid	14 ± 2	15 ± 2
Arginine	50 ± 4	41 ± 4
Aspartic acid	20 ± 3	11 ± 2
Cysteine	19 ± 5	15 ± 5
Glutamic acid	40 ± 3	24 ± 4
Glycine	41 ± 5	33 ± 5
Histidine	10 ± 2	< 5
Isoleucine	15 ± 3	16 ± 2
Leucine	19 ± 1	14 ± 1
Lysine	<15	<15
Methionine	5 ± 1	17 ± 6
Phenylalanine	7 ± 0.5	< 7
Serine	65 ± 3	29 ± 2
Taurine	208 ± 47	123 ± 20
Threonine	16 ± 3	8 ± 1
Tryptophane	4 ± 0.5	< 4
Tyrosine	11 ± 2	20 ± 2
Valine	20 ± 2	41 ± 4

Discussion

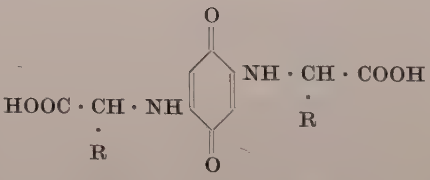
Appleman *et al.* (15) found that the decrease in the catalase content of the liver, which was observed in rats with Jensen sarcoma, is accompanied by an enlargement of the spleen and the liver of these animals. It is known that the liver catalase content is also strongly reduced in the livers of BY-fed rats (37). This could be the reason why the BY spleen changes in weight. It is more likely, however, that a simultaneous, partial inhibition of other enzymes and enzyme systems strongly influences the BY-spleen. Miller and Miller (16), for instance, have shown that amongst the in vivo degradation compounds of BY there are also *p*-phenylenediamine and its N-methyl derivatives. These substances are powerful enzyme inhibitors. They thus inhibit catalase (17), urease (18), succinoxidase (18), cozymase (19), yeast carboxylase (19), and some other SH-enzymes (20). However, Potter (20) showed that, of these compounds, only those possessing a quinoide structure are inhibitors of enzymes. This was later confirmed by Elson and Hoch-Ligeti (18). BY itself only inhibits cathepsin T (21). A great number of enzymes have also been found to be inhibited in the livers of BY-fed rats. Some of them are: catalase (37), cytochromoxidase (22), diaphorase (22), cathepsin (23), arginase (23), and xanthinoxidase (24). A high value of the liver phosphatase has been reported in BY-fed rats (23). The reduced content of free amino acids in the BY-spleen (Table 3) and in the BY-blood also points at an inhibition of other proteolytic enzymes.

If the idea is accepted that substances of a quinoide structure are the main enzyme inhibitors in BY-fed rats, we have to consider also possibilities for the occurrence of other quinoides. Among the known in vivo degradation products of BY is *p*-aminophenol (16) which might be converted to *p*-quinonimine and *p*-quinone (27) in the liver of the rat. The latter compound, which is a cancerogen itself (28), is a strong enzyme inhibitor (29).

The present writer has carried out a great number of analyses, but *p*-quinone could be detected neither in BY-spleens nor in BY-livers. If *p*-quinone were formed in vivo it might react with hemoglobine (30) or with free amino acids (31). The writer has investigated the reaction between α-amino acids and *p*-quinone, and substances of type I were isolated (32).



Type I



Type II

Dipeptides, however, show the ability of forming compounds of type II with *p*-quinone (32). Both these types of compounds are strongly red coloured and show strong absorption peaks in the ultra violet region of the spectrum. Therefore, if they were present in the BY-spleen or in the BY-liver, they ought to be easy to detect. In this laboratory a great number of BY-fed rats have been investigated, but no compounds of types I and II could be found in the livers or spleens. With α-amino acids *p*-quinonimine also gives compounds of type I (32). They possess the same properties as the *p*-quinone derivatives of type I, but could not be detected in the BY-organs mentioned. Under these circumstances it appears likely that the enzyme-inhibiting substance is *p*-quinondiimine rather than *p*-quinone or *p*-quinonimine.

In the investigation mentioned (32) it was found, furthermore, that substances of quinoide structure are easily adsorbed on casein. This finding might be an explanation of the fact that casein is a strong inhibitor of BY hepatoma development (33, 34).

It is known (38, 39) that compounds of quinoide structure are formed in vivo in rats with 3,4-benzpyrene and dibenzanthrazene sarcoma. Also in these cases strong inhibition of various liver enzymes takes place (22). One tumour inhibiting substance—4-dimethylaminostilbene—was found to *increase* the ability of the rat liver to synthesize enzymes (4). All these facts show that there is a strong experimental evidence for believing that substances of quinoide structure are responsible for the observed generally low enzyme contents of the hepatoma and of the surrounding liver tissue.

The observed low free amino acid content of the BY-spleen is probably not only a consequence of enzyme inhibition but also a consequence of a retardation of enzyme synthesis, as both essential and nonessential amino acids have been shown to have an influence on enzyme synthesis (35). The enlargement of the BY-spleen is thus looked upon as a lack-of-enzyme effect.

Hemine and globin synthesis, which to a great extent takes place in the rat spleen (36), can be influenced by the altered enzyme conditions of this organ. That this is the case has been shown experimentally (Table 2).

In this investigation 10 Wistar rats were fed with a diet containing *p*-phenylenediamine (15 mg per day and rat). No spleen enlargement or change in hemoglobine content of this organ could be observed even after 3 months feeding with the diet. This observation agrees with the finding that neither N_1N_1-dimethyl-*p*-phenylenediamine nor aniline are cancerogenous (25). These facts show that BY must be broken down *in the rat liver* to act as a cancerogen. Possibly the orally administered amines are inactivated by antibodies, for when BY itself is given to the animals an increase in the serum globulin content of the rat blood has been observed (26). In any case, it is obvious that the chemically very stable BY has a much greater chance to reach the rat liver unchanged than the reactive free amines administered. Relatively large amounts of BY can also easily be extracted from the BY-livers.

It seems as if especially the mitochondria of the rat liver cells undergo changes when BY is given orally to the animals (40); e.g., the ability of the mitochondria to synthesize certain enzymes is decreased (41).

The author is of the opinion that the data presented here support a theory by Seeger (42), according to which the development of cancer is believed to depend on irreversible changes in the red-ox processes of cells, caused by enzyme inhibition.

Summary

1. During oral administration of BY to rats their spleens increase in weight. The spleen weight reaches a maximum by the time of, or a little after, the development of the hepatoma and decreases thereafter.

2. The hemoglobine content of the spleen from BY-fed rats is strongly increased, thus provoking an abnormally deep colour of this organ.

3. The free amino acid content of the spleen from BY-fed rats is markedly changed, generally decreased (19 amino acids investigated).

4. The possibilities for compounds of quinoide structure to be the main enzyme inhibitors at the development of BY hepatoma are discussed.

This work was financially supported by a grant from *Lars Hiertas Minnes Fond.*

Institute for Research in Organic Chemistry, University of Stockholm.

REFERENCES

1. LOWENHAUPT, E., Cancer Research *9*, 121–6 (1949).
2. EDWARDS, J. E. and WHITE, J., J. Natl. Cancer Inst. *2*, 157–83 (1941).
3. VON EULER, H., Arkiv Kemi *43*, 469–76 (1953).
4. FEINSTEIN, R. N., Cancer Research *10*, 93–5 (1950).
5. GREENBERG, A. and ERICKSON, D., J. Biol. Chem. *156*, 679–82 (1944).
6. TAYLOR, A. and POLLAK, M. A., Cancer Research *2*, 223–7 (1942).
7. STRONG, L. C. and FRANCES, L. D., Am. J. Cancer *38*, 399–403 (1940).
8. ROBSCHEIT-ROBBINS, F. S. and WHIPPLE, G. H., J. Exptl. Med. *57*, 637–51 (1933).
9. SCHALES, O., Ber. *70 B*, 1874–80 (1937).
10. VON EULER, H. and HELLER, L., Arkiv Kemi *24 A*, No. 37 (1947).
11. REISS, M., Schwarz, L., and FLEISCHMANN, F., Z. physiol. Chem. *234*, 201 (1935).
12. SOLOMON, J. D., JOHNSON, C. A., SHEFFNER, A. L., and BERGHEIM, O., J. Biol. Chem. *189*, 629–35 (1951).
13. AWAPARA, J., Arch. Biochem. *19*, 172 (1948).
14. ÅKERFELDT, S., Acta Chem. Scand. *In press.*
15. APPLEMAN, D., SKAVINSKY, E. R., and STEIN, A. M., Cancer Research *10*, 498 (1950).
16. MILLER, J. A. and MILLER, E. C., Cancer Research *7*, 39–41 (1947).
17. DIEMAIR, W. and BOEKHOFF, K., Z. anal. Chem. *139*, 267–78 (1953).
18. ELSON, L. A. and HOCH-LIGETI, C., Biochem. J. *40*, 380–91 (1946).
19. KENSLER, C. J., DEXTER, S. O., and RHOADS, C. P., Cancer Resarch *2*, 1 (1942).
20. POTTER, V. R., Cancer Research *2*, 688–93 (1942).
21. SIEBERT, G., LANG, K., and WOLF, H., Biochem. Z. *322*, 446–55 (1952).
22. BERNHARD, P., Schweiz. Z. Biochem. *1*, 94–109 (1942).
23. WALDSCHMIDT-LEITZ, E., Problemy Biol. Med. *1935*, 615–18.
24. WESTERFELD, W. W., RICHERT, D. A., and HILFINGER, M. F., Cancer Research *3*, 486–94 (1950).
25. MUELLER, G. C., MILLER, J. A., and GLASSNER, M., J. Biol. Chem. *185*, 145 (1950).
26. HOCH-LIGETI, C., HOCH, H., and GOODALL, K., Brit. J. Cancer *3*, 140–7 (1949).
27. VON EULER, H., Arkiv Kemi. *6*, No. 29 (1953).
28. TAKIZAWA, N., Proc. Imp. Acad. (Tokyo) *16*, 309 (1940).
29. KUHN, R. and BEINERT, H., Ber. *76*, 904 (1943).
30. HEUBNER, B. W., Arch. exp. Pathol. Pharmacol. *72*, 239 (1913).
31. COOPER, E. A. and HAINES, R. B., Biochem. J. *22*, 317 (1928).
32. ÅKERFELDT, S., Arkiv Kemi. *7*, No 10 (1954).
33. KENSLER, C. J. *et al.*, Science *93*, 308–10 (1941).
34. MILLER, E. C. *et al.*, Cancer Research *5*, 713–16 (1945).
35. LITWACK, G., WILLIAMS, J. N., and ELVEHJEM, C. A., J. Biol. Chem. *201*, 261 (1953).
36. RICHMOND, J. E., ALTMAN, K. J., and SALOMON, K., J. Biol. Chem. *190*, 815–25 (1951).
37. VON EULER, H., HASSELQUIST, H., and ERIKSSON, E., Arkiv Kemi *4*, No. 39 (1952).
38. BERENBLUHM, J. and SCHOENTHAL, R., Cancer Research *6*, 699 (1946).
39. WOLF, G. and HEIDELBERGER, C. H., Cancer Research *11*, 290 (1951).
40. GOERNER, A., J. Biol. Chem. *122*, 529 (1938); ibid. *123*, 57 (1938); ibid. *128*, 559 (1939).
41. POTTER, V. R., PRICE, J. M., and MILLER, J. A., Cancer Research *10*, 650–8 (1950).
42. SEEGER, P. G., Z. Krebsforsch. *57*, 387–404 (1951).

Tryckt den 6 augusti 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 39

Communicated 10 March 1954 by Arne Fredga

Metalation and halogen-metal interconversion in the thiophene series

By Salo Gronowitz

With 4 figures in the text

Metalation has been found to take place preferentially at a position *ortho* to a substituent having an unshared electron pair, independently whether the substituent is *ortho*, *para* or *meta* directing in electrophilic aromatic substitution. This is best accounted for by assuming that the reaction involves an initial coordination of the metallic atom of the metalating agent with the unshared electron pair of the substituent or in the case of heterocyclic aromatic compounds with the unshared electron pair of the heterocyclic atom. Two contrary mechanisms have been proposed for the further reaction of the coordination complex. Morton (1) has suggested that the mechanism, after this initial coordination, involves mainly an electrophilic attack by the cationic part of the ion pair on the *ortho* position having the highest electron density, with the simultaneous removal of a proton by the carbanion. The second mechanism proposed by Roberts and Curtin (2) suggests that after the initial coordination the most acidic (most positively polarized) hydrogen atom is removed by the nucleophilic attack by the carbanion from the metalating agent. Both Roberts and Curtin (2) and Sunthankar and Gilman (3) have discussed the influence of the initial coordination step in facilitating the reaction in the *ortho* position.

The effect of the initial coordination may be illustrated by an example from thiophene chemistry. It is well known that electrophilic substitution takes place preferentially in the 2-position of thiophene and in the 3-position of thianaphthene. Both substances, however, are readily metalated in the 2-position. So it can be assumed that the initial step in the metalation of thiophenes is a coordination of the metal of the metalating agent with the unshared electron pair of the sulphur atom.

2-Thianaphthenecarboxylic acid is obtained in 82 % yield through the metalation of thianaphthene with *n*-butyllithium followed by carbonation. Hitherto the best method has been the direct reaction of thianaphthene with sodium powder in ether (4). The best yield obtained by this method, however, is 56 % and is very much dependent upon the dispersion of the sodium.

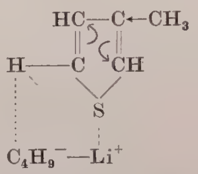
By studying the directing effect of different substituents in the 3-position of thiophene and their activating or deactivating effect on metalation, additional information about the mechanism of this reaction should be obtained. The present author has examined the metalation of 3-methylthiophene and the metalation and halogen-metal interconversion reaction of 3-bromothiophene with organolithium compounds.

The yields of the interconversion products were determined by the isolation of the acids obtained after carbonation of the organolithium compounds.

SCHICK and HARTOUGH (5) have metalated 3-methylthiophene in the 5-position with ethyl chloride and sodium. It has been found by the present author and by SICÉ (6) that *n*-butyllithium also metalates 3-methylthiophene in the 5-position.

BLANCHETTE and BROWN (7) were not able to reproduce the yields of SCHICK and HARTOUGH but found that the 3-methyl-5-thienylsodium first formed couples with the metalating agent in the sense of the Wurtz-Fittig reaction. The present author has not observed this complication with the corresponding lithium compound.

The metalation in the 5-position is to be expected if the reaction is formulated as a nucleophilic attack of the butyl carbanion on hydrogen.

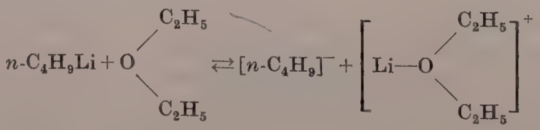


The inductive effect of the methyl group deactivates the whole thiophene nucleus towards nucleophilic attack by making all the hydrogens less acidic. The 5-(*meta*) position is, however, affected to a lesser extent than the 2-(*ortho*) position, and thus the butyl carbanion attacks the most acidic hydrogen in the position *meta* to the methyl group.

If the mechanism of the metalation involves an electrophilic substitution by the lithium cation it should take place in the 2-position where substitution is obtained in bromination, nitration and sulphonation of 3-methylthiophene.

It was found in a competitive experiment in which thiophene and 3-methylthiophene were metalated with insufficient amounts of *n*-butyllithium, by analysis of the infra red absorption spectrum, that the acid mixture obtained after carbonation consisted of about 75 % 2-thiophenecarboxylic acid and 25 % 3-methyl-5-thiophenecarboxylic acid. This indicates that thiophene is faster metalated and is more acidic than 3-methylthiophene.

MORTON *et al.* (8) have found that cumene is metalated by amylsodium in the *ortho* and *para* positions. It should be noted, however, that this reaction is carried out in cumene and not in ether. The influence of different solvents on the metalation reaction has not yet been thoroughly investigated. SCHICK and HARTOUGH (9) have found that 2-chlorothiophene undergoes different reactions in different solvents. They obtained 2-thienylsodium in benzene and 5-chloro-2-thienylsodium in ether. It is usually assumed that the catalytic effect of ethers on the metalation reaction depends upon their ability to coordinate the metalating agent and thus facilitate the polarization of the carbon metal bond. It is supposed that complete dissociation is obtained to a very small extent (10).

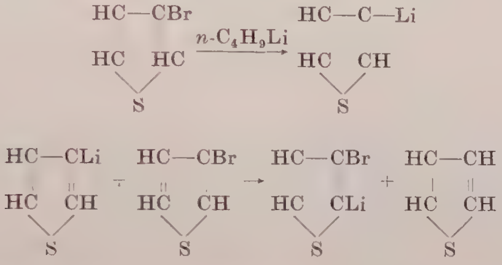


$$n\text{-}C_4H_9Li + O\begin{matrix}C_2H_5\\ \\ C_2H_5\end{matrix} \rightleftarrows [n\text{-}C_4H_9]^- + \left[Li{-}O\begin{matrix}C_2H_5\\ \\ C_2H_5\end{matrix}\right]^+$$

This effect cannot be obtained in aromatic hydrocarbons and it could thus be possible that a different mechanism is involved in this case. MORTON (11) has shown that the possibility of homolytic dissociation of amylsodium cannot be excluded.

3-Bromothiophene gives with phenyllithium after carbonation a bromothiophenecarboxylic acid in 71 % yield. This acid has the same infra red spectrum (Fig. 3) and gives no melting point depression with an authentic sample of 3-bromo-2-thiophenecarboxylic acid (12). Phenyllithium thus preferentially gives metalation instead of halogen-metal interconversion with 3-bromothiophene. The same acid, but always in yields smaller than 50 %, was obtained when n-butyllithium was allowed to react with 3-bromothiophene at room temperature, especially when a small excess of 3-bromothiophene was used. In addition thiophene was found in the reaction mixture. Thus the most probable mechanism is a two stage metalation.

The 3-thienyllithium first formed metalates 3-bromothiophene faster than the latter undergoes the halogen-metal interconversion reaction with n-butyllithium.



A similar mechanism is proposed by GILMAN *et al.* for the reactions of 2-bromodibenzofuran and 3-bromodibenzofuran and for the *para* and *meta* brominated anisols with n-butyllithium (13).

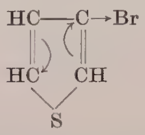
The yield (over 50 %) rules out a complete two stage process with phenyllithium. This tendency of aromatic lithium compounds to give metalation instead of halogen-lithium interconversion has previously been observed with p-bromoanisol (13).

The present author found, however, that when 3-bromothiophene was added to a slight excess of n-butyllithium at $-70°$, and carbonated after a short reaction period, 3-thiophenecarboxylic acid was obtained in 79 % yield. It has recently been shown (14) that 3-bromothiophene can be easily synthesized, and as the organolithium-compound readily undergoes all the reactions of the Grignard reagents, 3-thienyllithium can be used for the synthesis of 3-substituted derivatives of thiophene. 3-Bromothiophene did not react with phenyllithium at this temperature.

GILMAN *et al.* (15) have found that rearrangement occurs during the halogen-metal interconversion of 3-bromobenzofuran to the corresponding 2-isomer. Analysis of the infra red absorption spectrum shows, however, that no such rearrangement from 3-thienyllithium to 2-thienyllithium occurs under the experimental conditions investigated.

In order to prove that no 2-thiophenecarboxylic acid was formed during the reaction, the spectra of pure 2-thiophenecarboxylic acid, 3-thiophenecarboxylic acid (obtained via the Grignard reagent) and of the reaction products were compared. It was found that there were two absorption maxima in 2-thiophenecarboxylic acid at 7.37 μ and 11.60 μ which were absent in pure 3-thiophenecarboxylic acid. These maxima were also absent in the products obtained in the reactions between 3-bromothiophene and *n*-butyllithium. A third strong maximum at 9.65 μ in 2-thiophenecarboxylic acid is not entirely absent in the spectrum of the reaction products. 3-Thiophenecarboxylic acid obtained via the Grignard reagent, however, also shows a weak maximum at 9.65 μ. It is therefore possible that 3-thiophenecarboxylic acid has a weak maximum at this wavelength (Fig. 4).

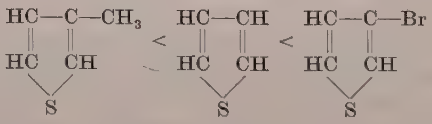
The ready metalation in the 2-position is to be expected. The strong inductive effect of the bromine atom makes the hydrogen in the 2-(*ortho*) position more acidic than in the 5-(*meta*) position and makes all the hydrogens more acidic than in thiophene (cf. bromoacetic acid and acetic acid). 3-Bromothiophene seems to be metalated much faster than thiophene. If 3-bromothiophene is treated for half an hour with *n*-butyllithium at −30° infra red absorption analysis indicates that after carbonation appreciable amounts of 3-bromo-2-thiophenecarboxylic acid are formed. That 3-bromothiophene is more acidic than thiophene is also evident from the fact that 3-thienyllithium metalates 3-bromothiophene.



It has thus been found that the orientation and reaction rates in the metalation of 3-methylthiophene and 3-bromothiophene are those that would be predicted if the inductive effect was most important in determining the reaction. The importance of the inductive effect has previously been pointed out by ROBERTS and CURTIN (2) and WITTIG (16).

This is in accordance with the view of INGOLD (17) that the tautomeric (resonance) effect of halogens is a polarisability effect, which is developed essentially in the course of reaction and arises from the demand of the reagent. The tautomeric effect of the bromine atom would tend to counteract the reaction if it is formulated as a nucleophilic attack on hydrogen. There is thus no need to take it into consideration, for a polarisability effect will always be such as to facilitate reaction.

It has thus been shown that 3-methylthiophene and 3-bromothiophene are metalated in different α-positions and that the metalation velocity and acidity increases in the following series



These experimental results are in accordance with the views held by ROBERTS and CURTIN and by SUNTHANKAR and GILMAN on the mechanism of the metalation reaction. But it seems to be more difficult, in this case, to interpret the results according to MORTON.

Experimental

All the reactions were carried out in the usual Grignard apparatus in a nitrogen atmosphere. *n*-Butyllithium and phenyllithium were prepared according to WITTIG (18).

3-Methyl-5-thiophenecarboxylic acid. To a solution of 10 g of 3-methylthiophene in 50 ml of absolute ether was added, in a slow stream, 100 ml of an approximately 1-molar solution of *n*-butyllithium in ether. The reaction mixture was refluxed for two hours and allowed to stand at room temperature overnight. The mixture was then poured on a slurry of Dry Ice and ether. When the Dry Ice had evaporated the mixture was hydrolized with water. The aqueous phase was separated, and the ether layer extracted several times with dilute sodium hydroxide. The combined aqueous layers were acidified in the cold with 1:1 hydrochloric acid precipitating 9.7 g crude 3-methyl-5-thiophenecarboxylic acid. The acid was first recrystallized from hot water, decolorized with charcoal and then recrystallized from petrol, yielding 8.8 g of pure white acid. M.p. 120°–122°. Yield 68 %.

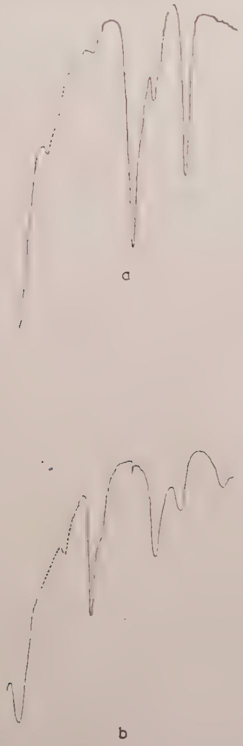


Fig. 1.

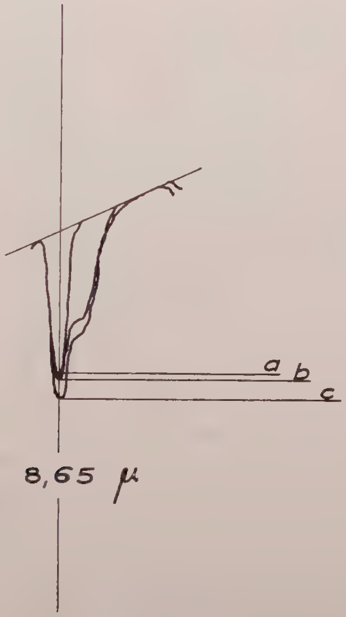


Fig. 2.

Fig. 1. *a*. 2-Thiophenecarboxylic acid in chloroform. *b*. 3-Methyl-5-thiophenecarboxylic acid in chloroform.

Fig. 2. *a*. 12.8 mg of 3-methyl-5-thiophenecarboxylic acid in 1 ml of chloroform. *b*. 59.6 mg of the acid mixture in 1 ml of chloroform. *c*. 16.1 mg of 3-methyl-5-thiophenecarboxylic acid in 1 ml of chloroform.

Competitive metalation of thiophene and 3-methylthiophene. To 8.4 g of thiophene and 9.8 g of 3-methylthiophene in 100 ml of absolute ether was added 95 ml of an approximately 1-molar solution of *n*-butyllithium. The mixture was refluxed for four hours, carbonated and worked up as above yielding 8.1 g of acidic material m.p.

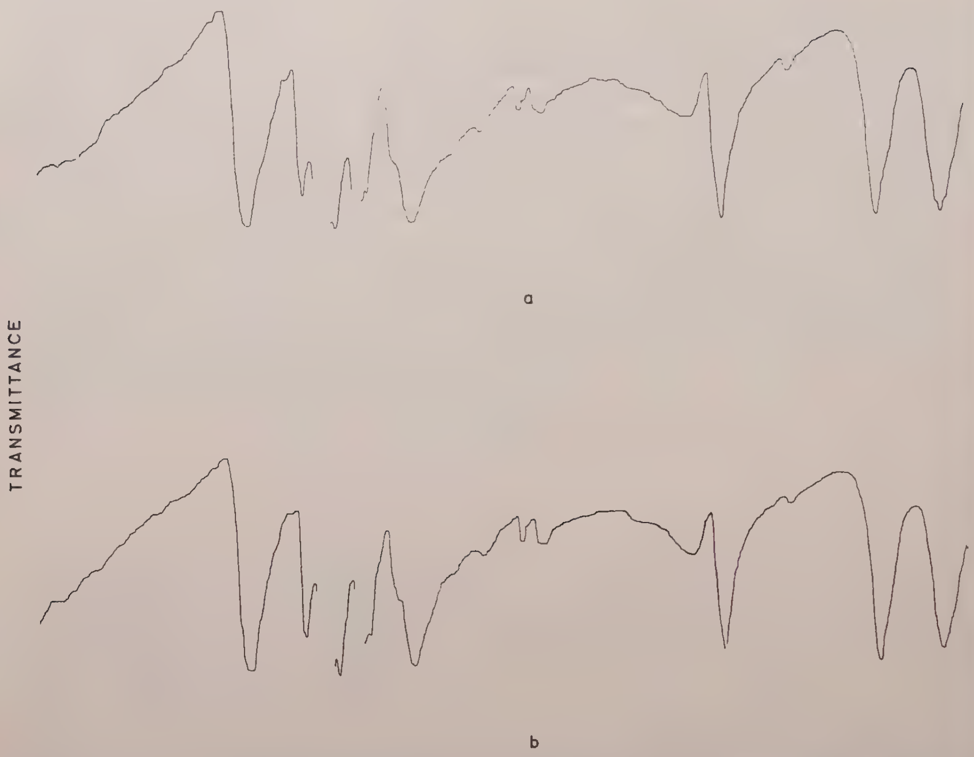


Fig. 3. *a*. 3-Bromo-2-thiophenecarboxylic acid, via the Grignard reagent of 2,3-dibromothiophene, as nujol mull. *b*. 3-Bromo-2-thiophenecarboxylic acid, via metalation of 3-bromothiophene, as nujol mull.

90°–120°. Infra red absorption analysis showed that the mixture consisted of about 75 % 2-thiophenecarboxylic acid and 25 % of 3-methyl-5-thiophenecarboxylic acid. For the analysis of the mixture the region between 8–10 μ was used. In this region each substance has a characteristic absorption maximum, 3-methyl-5-thiophenecarboxylic acid at 8.65 μ and 2-thiophenecarboxylic acid at 9.07 μ (Fig. 1). The amount of 3-methyl-5-thiophenecarboxylic acid in the mixture was determined by comparison with solutions of known concentrations (Fig. 2). In the spectrum of the mixture no maxima were present which could not be assigned to either 2-thiophenecarboxylic acid or 3-methyl-5-thiophenecarboxylic acid. Thus it can be assumed that the mixture contained no other substance in appreciable amounts.

2-Thianaphthenecarboxylic acid. 15 g of thianaphthene in 100 ml of absolute ether was refluxed for two hours with 100 ml of an approximately 1-molar solution of *n*-butyllithium and allowed to stand overnight at room temperature before carbonation. The products were worked up in the usual manner yielding 14.2 g of 2-thianaphthenecarboxylic acid. 2.0 g of thianaphthene was recovered from the ether phase. The acid was recrystallized from methanol and water. M.p. 234°–236°. Yield 82 %.

Metalation of 3-bromothiophene with phenyllithium. To 4 g of 3-bromothiophene in 25 ml of absolute ether was added 0.025 moles of phenyllithium in 25 ml of absolute

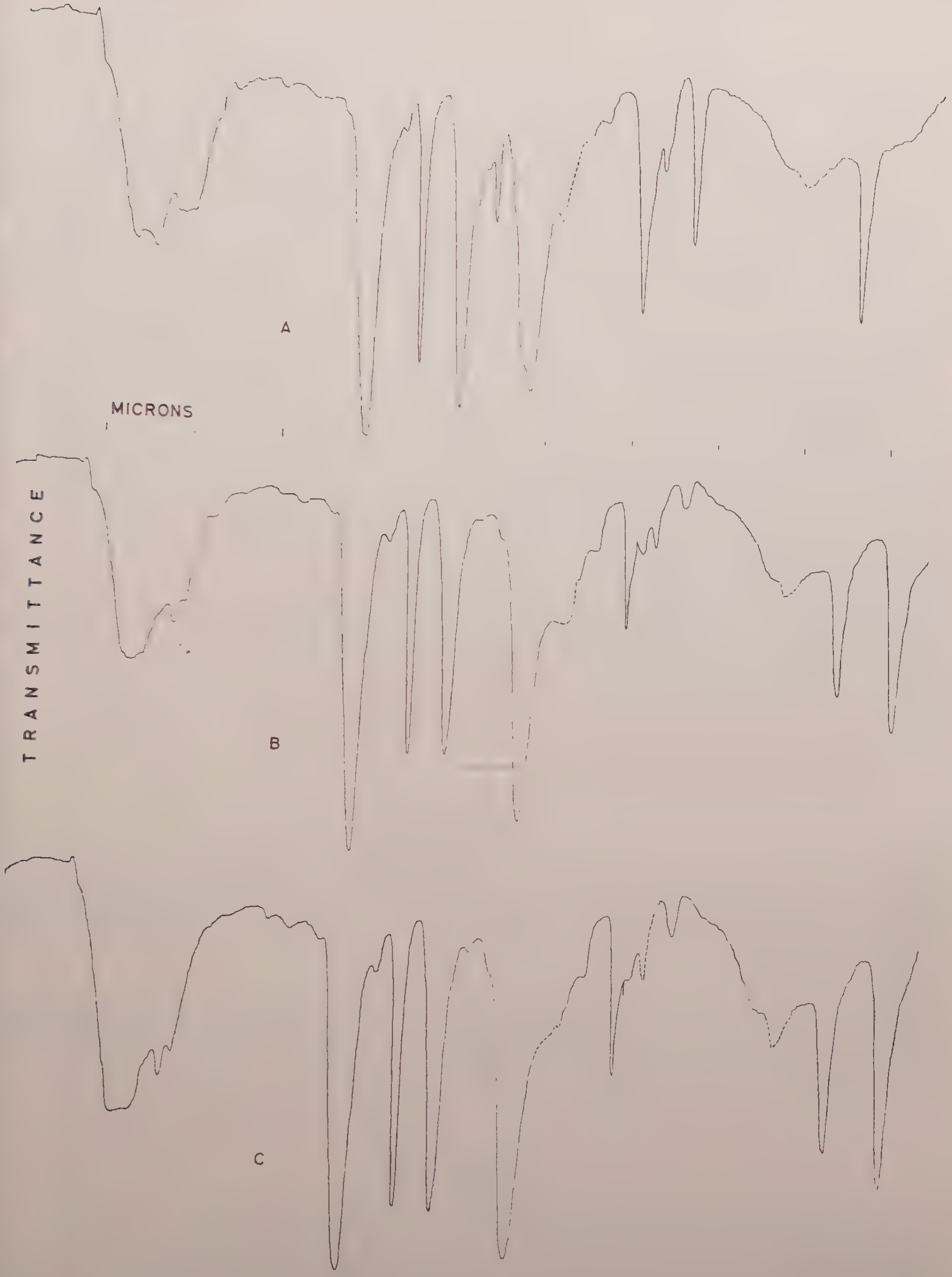


Fig. 4. *A*. 2-Thiophenecarboxylic acid in chloroform. *B*. 3-Thiophenecarboxylic acid, via the Grignard reagent of 3-bromothiophene, in chloroform. *C*. 3-Thiophenecarboxylic acid, via 3-thienyllithium, in chloroform.

ether. The mixture was allowed to stand overnight and was then carbonated and worked up in the usual manner yielding 3.6 g of 3-bromo-2-thiophenecarboxylic acid. The acid was recrystallized from hot water. M.p. 195°–197°. Yield 72 %.

Metalation of 3-bromothiophene with n-butyllithium. To a solution of 8.1 g of 3-bromothiophene in 50 ml of absolute ether was added 45 ml of an approximately 1-molar ether solution of *n*-butyllithium. The mixture was allowed to stand for three hours before carbonation and after the usual treatment yielded 3.7 g of 3-bromo-2-thiophenecarboxylic acid. M.p. 195°–197°. Yield 36 %. The ether phase was dried and fractionated and the fraction boiling between 80°–100°, consisting of thiophene and *n*-butylbromide, was treated with DENIGÉ's reagent (19) in excess in order to precipitate the thiophene as a complex with basic mercuric sulphate. 14.3 g of the complex was obtained which is equivalent to 1.1 g of thiophene. Yield of thiophene 26 %.

3-Thiophenecarboxylic acid. To a solution of 0.07 moles of *n*-butyllithium in 70 ml of absolute ether cooled to $-70°$, 8.1 g of 3-bromothiophene in 50 ml of absolute ether also cooled to $-70°$ was added in a slow stream. The mixture was stirred for ten minutes at this temperature and was then carbonated and worked up as usual. 5.0 g of 3-thiophenecarboxylic acid was obtained. The acid was recrystallized once from hot water. M.p. 138°–139°. Yield 79 %. The acid gave no melting point depression with an authentic sample of 3-thiophenecarboxylic acid and gave the same infra red absorption spectrum (Fig. 4).

If this reaction was carried out at higher temperatures and especially for longer periods mixtures of 3-thiophenecarboxylic acid and 3-bromo-2-thiophenecarboxylic acid were obtained. The infra red absorption spectra showed in addition to the maxima of 3-thiophenecarboxylic acid, the characteristic maxima of 3-bromo-2-thiophenecarboxylic acid.

The infra red spectra were recorded with a Perkin-Elmer mod 21 spectrophotometer equipped with NaCl prism.

Summary

Metalation and the halogen-metal interconversion reaction with 3-methylthiophene and 3-bromothiophene has been investigated. The halogen-metal interconversion reaction between 3-bromothiophene and *n*-butyllithium at low temperatures opens a new route to 3-substituted derivatives of thiophene.

The author wishes to express his thanks to Professor ARNE FREDGA for valuable discussions. Thanks are also due to Mr. A. ROSENBERG for taking the infra red spectra.

Department of Organic Chemistry, Chemical Institute, University of Uppsala, March 1954.

REFERENCES

1. MORTON, A. A., J. Am. Chem. Soc. *69*, 969 (1947).
2. ROBERTS, J. D., and CURTIN, D. Y., J. Am. Chem. Soc. *68*, 1658 (1946).
3. SUNTHANKAR, S. V., and GILMAN, H., J. Org. Chem. *16*, 8 (1951).
4. SCHÖNBERG, A., PETERSEN, E., and KALTSCHMITT, H., Ber. *66*, 233 (1933).
5. SCHICK, J. W., and HARTOUGH, H. D., J. Am. Chem. Soc. *70*, 1645 (1948).
6. SICÉ, J., J. Org. Chem. *19*, 70 (1954).
7. BLANCHETTE, J. A., and BROWN, E. V., J. Am. Chem. Soc. *74*, 1849 (1952).
8. MORTON, A. A., MASSENGALE, J. T., and BROWN, M. L., J. Am. Chem. Soc. *67*, 1620 (1945).

9. Schick, J. W., and Hartough, H. D., J. Am. Chem. Soc. *70*, 286 (1948).
10. Wittig, G., Naturwiss. *30*, 698 (1942).
11. Morton, A. A., and Cluff, E. F., J. Am. Chem. Soc. *74*, 4056 (1952).
12. Steinkopf, W., and Köhler, W., Ann *532*, 250 (1937). Steinkopf, W., Jacob, H., and Penz, H., Ann. *512*, 137 (1934).
13. Gilman, H., Langham, W., and Moore, F. W., J. Am. Chem. Soc. *62*, 2327 (1940).
14. Gronowitz, S., Arkiv Kemi, *in print.*
15. Gilman, H., and Melstrom, D. S., J. Am. Chem. Soc. *70*, 1655 (1948).
16. Wittig, G., Angew. Chem *66*, 10 (1954).
17. Ingold C. K., and Shaw, F. R., Soc. *1949*, 575.
18. Wittig, G., Angew. Chem. *53*, 243 (1940).
19. Denigé, G., Compt. rend. *120*, 781 (1895).

Tryckt den 6 augusti 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 40

Read 26 May 1954

X-ray studies of sulfides of titanium, zirconium, niobium, and tantalum

By G. Hägg and N. Schönberg

With 1 figure in the text

Introduction

Among the sulfides of the metals Ti, Zr, Nb, and Ta the existence of the compounds TiS (1), TiS_2 (2), ZrS_2 (3) and NbS (1) has been confirmed by structure determinations. The present investigation was started in order to throw light on the relationship between the metal-sulfur systems, where the metal is any of the above-mentioned transition elements.

Experimental

The powdered metals used as starting materials were of fairly high purity, about 99.5 %. The sulfides were prepared in two different ways:

A. The two elements were mixed in different proportions, and the mixtures were heatad in evacuated and sealed silica tubes at about 750° C for up to two weeks.

B. A steady stream of dry H_2S was passed over the metals at various temperatures between 550° C and 900° C.

The phase analysis was made from powder photographs taken with Cu-*Kα* radiation, using a camera of the Guinier type with a bent quartz monochromator.

The unit cell dimensions and structure types of the phases are listed in Table 7 and the interatomic distances in Table 8.

The structure of the sulfide phases

The Ti-S system

Goldschmidt (2) reported in 1927 that TiS_2 has the $Cd(OH)_2$ structure (C 6 type). One of the present authors (N.S.) has recently found the monosulfide TiS (1) to be isomorphous with NiAs (B 8 type). Biltz, Ehrlich and Meisel (4) investigated the Ti-S system by tensimetric analysis and X-ray methods and proposed the existence of the following phases: a subsulfide $TiS_{0.2-<1}$, a monosulfide $TiS_{1.0}$, a sesquisulfide $TiS_{1.1-1.5}$, a disulfide $TiS_{\sim 1.7-2}$, and a trisulfide TiS_3.

No traces of the two phases with the compositions $TiS_{1.1-1.5}$ and TiS_3 could be found in the powder photographs in the present investigation, but a subsulfide with unknown structure and with the approximate composition $TiS_{0.5}$ seems to exist. The homogeneity range of TiS_2 was not determined but seems to be smaller than that given by BILTZ *et al.* (4).

The Zr-S system

Already in 1924 VAN ARKEL (3) found ZrS_2 to be of the C 6 type. STROTZER, BILTZ and MEISEL (5) reported the following phases in the Zr-S system: a subsulfide in the $ZrS_{0.20-0.33}$ interval, a "0.75-type" sulfide in the $ZrS_{0.5-0.9}$ interval, a sesquisulfide $ZrS_{1.2-1.5}$, a disulfide $ZrS_{1.8-2.0}$, and a trisulfide $ZrS_{2.8-3.2}$.

In the present study a subsulfide of undetermined structure and composition was found to exist besides the ZrS and ZrS_2 phases wich both have narrow homogeneity ranges.

The ZrS phase is homogeneous at the composition 1:1 and is of the B 11 type, i.e. isomorphous with the intermetallic phases γ-CuTi (6) and AgZr (7). The space-group is $D_{4h}^{7} - \mathrm{P}\ 4/n\,m\,m$ with the atomic grouping: 2 Zr in 2(c) $0, \frac{1}{2}, z$; $\frac{1}{2}, 0, \bar{z}$ and 2 S in the same position. A systematic variation of the z values for the Zr and S atoms gave the best agreement between the calculated and observed intensities, when $z_{Zr} = 0.19$ and $z_S = 0.69$ (Table 1). It is remarkable that $|z_{Zr} - z_S| = \frac{1}{2}$, giving rise to identical Zr and S lattices.

The Nb-S system

According to BILTZ and KÖCHER (8) there exist two intermediate phases in the Nb-S system, viz., a phase characterized as a sesquisulfide Nb_2S_3 but having a very broad homogenity range up to the approximate composition NbS_4, and a monosulfide NbS which is homogeneous in the $NbS_{0.5-1.0}$ interval. No structures were given by the two authors.

Two sulfide phases NbS and NbS_2 with rather small homogeneity ranges, were also found to exist in the present study. Because of the impossibility of preparing NbS in a pure state, the homogeneity limits could not be determined with any certainty but seem to be approximately $NbS_{0.9}$ and $NbS_{1.2}$. For NbS in equilibrium with Nb the WC structure was observed and for the phase with an excess of sulfur the NiAs structure (1). The NbS_2 phase does not exist with a greater sulfur content than corresponding to the composition 1:2 and has a narrow homogeneity range—probably smaller than 1 atomic %.

The disulfide NbS_2 was found to be of the rhombohedral C 19 type ($CdCl_2$ structure), common for the dichlorides of the metals Mg, Zn, Cd, Mn, Fe, Co, and Ni. The atoms are located in the following atomic positions of the space-group $D_{3d}^{5} - R\,\bar{3}\,m$: 1 Nb in 1 ($a$) 0,0,0 and 2 S in 2 ($c$) x, x, x; $\bar{x}, \bar{x}, \bar{x}$ with $x = 0.25$. For the value of the rhombohedral angle $\alpha = 33.33°$ the sulfur atoms will be arranged in an ideal cubic close-packing; the actual value is $\alpha = 30.95°$.

The Ta-S system

BILTZ and KÖCHER (9) investigated the Ta-S system by dilatometric and X-ray methods. According to these authors the system is very complicated. The

Table 1. Intensities calculated and observed for the ZrS phase

h k l	$I_{\text{calc.}}$	$I_{\text{obs.}}$	*h k l*	$I_{\text{calc.}}$	$I_{\text{obs.}}$
0,0,1	31	35	3,0,3	2	0
0,0,2	123	110	2,0,5	1	0
1,0,1	179	160	1,0,6	41	50
1,1,0	234	230	3,1,3	24	25
1,0,2	168	160	2,1,5	5	0
1,1,1	11	10	2,2,4	0	0
0,0,3	14	15	3,2,1	28	25
1,1,2	120	110	1,1,6	24	25
1,0,3	11	10	3,0,4	57	50
2,0,0	94	110	3,2,2	52	50
2,0,1	0	0	3,1,4	0	0
1,1,3	29	25	2,0,6	21	25
0,0,4	0	0	0,0,7	1	0
2,0,2	77	75	3,2,3	4	0
2,1,1	58	50	2,2,5	11	0
1,0,4	134	110	4,0,0	24	25
2,1,2	115	110	4,0,1	2	0
2,0,3	22	25	2,1,6	60	75
1,1,4	0	0	1,0,7	9	0
2,1,3	6	10	3,0,5	0	0
0,0,5	5	0	4,0,2	28	25
2,2,0	50	50	4,1,1	23	25
2,2,1	3	0	1,1,7	2	0
1,0,5	2	0	3,1,5	26	25
2,0,4	0	0	3,3,0	30	25
2,2,2	41	35	3,2,4	117	110
3,0,1	17	15	4,1,2	53	50
1,1,5	70	50	3,3,1	3	0
3,1,0	77	75	4,0,3	12	0
2,1,4	154	160	3,3,2	39	50
3,0,2	34	35	2,2,6	34	25
3,1,1	5	0	2,0,7	4	0
2,2,3	12	10	4,1,3	9	0
3,1,2	77	75	4,2,0	101	75
0,0,6	7	0			

intermediate phases reported by Biltz and Köcher were a subsulfide $\mathrm{TaS}_{0.3-\sim 1.0}$, a "sulfur-rich subsulfide" $\mathrm{TaS}_{\sim 1.0-\sim 1.9}$, a disulfide TaS_2 which under certain conditions can dissolve sulfur up to the approximate composition TaS_3, a trisulfide TaS_3 with a very broad homogeneity range, TaS_{3-7}. Biltz and Köcher found that TaS_2 was hexagonal ($a = 3.40$ Å, $c = 5.90$ Å, $c/a = 1.74$) and proposed a structure belonging to the $\mathrm{Cd(OH)_2}$ type (C 6) or the MoS_2 type (C 7) or possibly another lattice type. The reported observed intensities are, however, only in agreement with the C 6 type.

Our results agree with those given by Biltz and Köcher only in one point, viz., the existence of a disulfide of the $\mathrm{Cd(OH)_2}$ structure. No intermediate phase in the Ta-TaS_2 interval was found to exist, and the use of sulfur in great excess only caused the formation of disulfide and free sulfur.

Four different modifications of TaS_2 were obtained, all of them having layer lattices: α (C 6 type), β (C 27 type), γ (C 19 type) and δ (new structure type).

While α-TaS_2 has a comparatively broad homogeneity range (probably about 5 atomic %) no deviations from the stoichiometric composition 1:2 were found for the other three modifications.

Only α-TaS_2 could be prepared in a pure state, while the other three TaS_2 phases could not be entirely separated from each other. The unit cell dimensions of α-TaS_2 were found to vary continuously between $a = 3.319$ Å, $c = 6.275$ Å, $c/a = 1.891$ ($V = 59.87$ Å^3) and $a = 3{,}346$ Å, $c = 5.860$ Å, $c/a = 1.751$ ($V = 56.80$ Å^3). The former lattice constants correspond to the sulfur-poor phase limit at the approximate composition $TaS_{1.6}$. This limit could not be determined with any accuracy because of the difficulty met with in transforming all the metal into a sulfide with a lower sulfur content than that corresponding to the stoiciometric composition TaS_2. At the composition TaS_2 the atomic positions of the α-TaS_2 phase are: 1 Ta in 1(a) 0,0,0 and 2 S in 2(d) $^1/_3$, $^2/_3$, z; $^2/_3$, $^1/_3$, $\bar{z}$ with $z \sim 0.25$ in the space-group $D_{3d}^3 - P\,\bar{3}\,m$. It is evident from density determinations and from the diffraction intensities that the atomic arrangement of α-TaS_2 at a composition TaS_x, where $x < 2$, ought to be described as: 1 Ta in 1(a) 0,0,0, $(2 - x)/x$ Ta in 1(b) 0,0, $\frac{1}{2}$ and 2 S in 2(d) $^1/_3$, $^2/_3$, $^1/_4$; $^2/_3$, $^1/_3$, $^3/_4$. For the theoretical value of $x = 1$ the NiAs structure is attained. But this limit is not reached in the present case.

The four tantalum disulfide modifications seem all to exist at the composition TaS_2. It was not possible to transform one phase into another by means of heat-treatments at different temperatures. With the synthesis in evacuated and sealed silica tubes the four phases were obtained in varying amounts although the experimental conditions were the same, as far as it was possible to control. No traces of δ-TaS_2 were obtained in samples prepared from Ta and H_2S. As an increasing amount of the most complicated TaS_2 modification (δ) was obtained at prolonged reaction times, this phase is probably the most stable one. The close similarity between the structures of the four phases makes it very probable, however, that their energies differ very sligthly.

If hexagonal crystal axes are chosen for δ-TaS_2, the unit cell contains six formula units TaS_2 with the following atomic positions in the space-group $D_{3d}^5 - R\,\bar{3}\,m$:

6 Ta in 6(c) 0,0,$\bar{z}$; with $z = {}^1/_{12}$,
6 S in 6(c) with $z = {}^5/_{24}$,
6 S in 6(c) with $z = {}^3/_8$.

In order to show the relation between the four TaS_2 modifications Fig. 1 has been drawn, where the positions of the atoms in the 110 planes are given to characterize the atomic groupings. If the three projections of the positions on the basal planes are designated by A, B and C, the tantalum lattice can be symbolized by the sequences A . . ., AB . . ., ABC . . ., AABBCC . . . for the α-, β-, γ- and δ-TaS_2 phases respectively. In the two latter cases the atoms have a rhombohedral symmetry.

Because of the great difference between the atomic factors of Ta and S it is fairly difficult to determine the configuration of the S lattice from X-ray photograps. The observed intensities are in good agreement with those calculated for the given structures, however, as is evident in Tables 2, 3, 4 and 5.

In the cases of TiS_2 and NbS_2 the positions given for the S atoms are reliable, and for isostructural reasons α-TaS_2 and γ-TaS_2 are undoubtly of the C 6

Table 2. Intensities calculated and observed for the α-TaS_2 phase

$h\,k\,l$	$I_{\text{calc.}}$	$I_{\text{obs.}}$	$h\,k\,l$	$I_{\text{calc.}}$	$I_{\text{obs.}}$
0,0,1	419	400	2,0,0	40	50
0,0,2	38	50	2,0,1	120	140
1,0,0	187	230	1,0,4	34	30
1,0,1	428	400	2,0,2	70	50
1,0,2	148	140	1,1,3	93	80
0,0,3	30	30	0,0,5	12	10
1,1,0	118	140	2,0,3	76	50
1,0,3	136	140	1,1,4	115	140
1,1,1	125	140	2,1,0	43	50
0,0,4	35	30	1,0,5	69	80
1,1,2	50	50			

Table 3. Intensities calculated and observed for the β-TaS_2 phase

$h\,k\,l$	$I_{\text{calc.}}$	$I_{\text{obs.}}$	$h\,k\,l$	$I_{\text{calc.}}$	$I_{\text{obs.}}$
0,0,2	175	200	2,0,0	4	5
0,0,4	18	15	2,0,1	42	40
1,0,0	17	15	2,0,2	14	15
1,0,1	201	200	1,0,8	7	0
1,0,2	50	40	2,0,3	25	25
1,0,3	72	70	2,0,4	13	15
1,0,4	31	25	1,1,6	37	40
0,0,6	14	15	2,0,5	20	40
1,0,5	39	40	1,0,9	32	
1,0,6	16	15	0,0,10	6	0
1,1,0	47	70	2,0,6	7	0
1,1,2	50	40	1,0,10	8	0
0,0,8	14	15	1,1,8	49	40
1,0,7	46	40	2,0,7	25	25
1,1,4	20	25			

Table 4. Intensities calculated and observed for the γ-TaS_2 phase

$h\,k\,l$	$I_{\text{calc.}}$	$I_{\text{obs.}}$	$h\,k\,l$	$I_{\text{calc.}}$	$I_{\text{obs.}}$
0,0,3	411	400	1,0,11	52	50
0,0,6	34	30	2,0,1	52	50
1,0,1	221	230	2,0,2	21	15
1,0,2	82	80	2,0,4	88	80
1,0,4	271	230	2,0,5	48	50
1,0,5	121	140	1,1,9	91	80
0,0,9	31	30	1,0,13	42	30
1,0,7	82	80	2,0,7	42	30
1,0,8	126	140	0,0,15	13	10
1,1,0	106	140	2,0,8	65	50
1,1,3	112	140	1,0,14	15	10
1,0,10	24	15	2,0,10	54	50
0,0,12	32	30	1,1,12	109	80
1,1,6	44	50			

Table 5. Intensities calculated and observed for the δ-TaS_2 phase

h k l	$I_{calc.}$	$I_{obs.}$	*h k l*	$I_{calc.}$	$I_{obs.}$
0,0,6	151	150	2,0,4	7	5
0,0,12	13	15	1,0,22	2	5
1,0,1	59	50	2,0,5	7	
1,0,2	10	10	2,0,7	26	25
1,0,4	28	25	2,0,8	3	10
1,0,5	24	25	1,0,23	12	
1,0,7	87	90	2,0,10	8	5
1,0,8	10	10	2,0,11	17	15
1,0,10	20	25	1,0,25	10	10
1,0,11	38	50	2,0,13	15	50
1,0,13	31	50	1,1,18	33	
0,0,18	34		2,0,14	7	0
1,0,14	14	15	1,0,26	7	0
1,0,16	4	5	2,0,16	3	0
1,0,17	33	25	0,0,30	4	0
1,1,0	42	50	2,0,17	18	15
1,1,6	45	50	1,0,28	5	0
1,0,19	8	5	2,0,19	4	0
1,0,20	7	5	1,0,29	4	0
0,0,24	13	15	2,0,20	4	0
1,1,12	18	15	1,1,24	43	50
2,0,1	13	15	2,1,1	14	10
2,0,2	3	0	2,1,2	3	

and C 19 types respectively. The interatomic S-S distances—close to 3.4 Å—further support these structures, as the deviations from this value hitherto observed for metal sulfides are very small. We therefore also assume analogous close-packed arrangements of S atoms for β-TaS_2* and δ-TaS_2 as the most likely ones since good agreement is obtained between the observed intensities of the diffraction lines and the $p\,|F|^2$ values calculated for these groupings.

The crystal lattices of the disulfides are built up from TaS_2 layers so that the S atoms are arranged in a hexagonal (α), or a cubic close-packing (γ), or a mixture of both (β, δ).

These structures can be completely described in a very simple way by using

---

* The MoS_2 and WS_2 phases are reported to be of the C 7 type. The metal atoms have the same arrangement in the lattices of the C 27 and C 7 types, but the sulfur atoms have the stacking sequence ABAC in the former structure and AABB in the latter one. The difference between the $p\,|F|^2$ values calculated for the two lattice types is of such an order for some reflections with rather small deviation angles that it is possible to distinguish the structures from each other even in the cases of the heavy metals Ta and W. Photographs taken from different MoS_2 and WS_2 samples showed so large disagreements between the observed line intensities and those calculated for the C 7 type that one may assume that the grouping of sulfur atoms occurs in other sequences than AABB. As the reflections with $l \neq 0$ were generally very diffuse it was not possible to decide whether the disagreements mentioned are due to stacking disorder of the C 7 structure or if the sulfur atoms have, for instance, a close-packed arrangement (C 27 type). The fact that the unusually small S-S distances, which are obtained for MoS_2 and WS_2 with the C 7 structure, will not be changed by a stacking disorder, speaks in favour of the C 27 type.

The same situation also characterizes the ReS_2 phase.

A more thorough study of this problem will be started within a short time.

Table 6. Structure types of the TaS_2 modifications

Phase	Type	Notation 1	Notation 2
α	C 6	(+)−	A
β	C 27	(+)+(−)−	AB
γ	C 19	(+)+	ACB
δ	New	(+)−(+)+	AACCBB

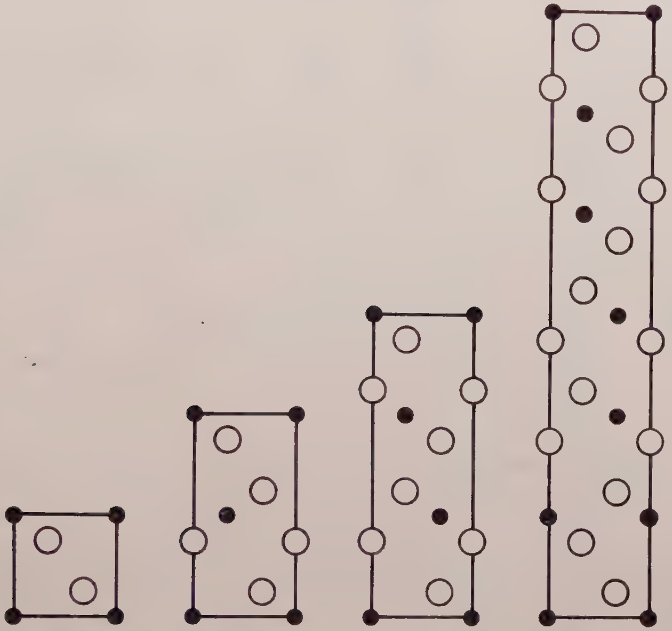

α (C 6) β (C 27) γ (C 19) δ (new)

Fig. 1. The positions of the atoms in the 110 planes of the four TaS_2 modifications. ● = Ta. ○ = S.

a notation proposed by one of present authors (G.H.) in 1942 (10). (This proposal seems to have been overlooked by many authors and the same notation was later proposed by NABARRO and, with a formal change, by FRANK in 1951 (11). The still more contracted symbols proposed by SHDANOV (12) in 1945 and by RAMSDELL (13) in 1947 were based on the same idea but are not quite suitable for the present purpose.) In this notation the relationship between a pair of neighbouring layers in a close-packing is denoted by + if the passage from one plane to the next involves the translation $^{1}/_{3}$, $^{2}/_{3}$ in the basal plane. If the translation for a passage in the same direction is $^{1}/_{3}$, $^{2}/_{3} = {}^{2}/_{3}$, $^{1}/_{3}$ the sign − is used. If there is an intervening metal or cation layer the corresponding sign is put in a parenthesis. The symbols for the four TaS_2 phases according to this notation |1| are given in Table 6. Naturally, all signs in a given symbol can be reversed simultaneously.

Table 7. Structure types and unit cell dimensions of the monosulfides and disulfides in the Ti-S, Zr-S, Nb-S and Ta-S systems

Phase	Structure type	Z	a, Å	c, Å	c/a	V, Å³	Density calc.	Density obs.
TiS	B 8	2	3.30	6.44	1.95	60.58	4.38	4.20
TiS_2	C 6	1	3.39	5.70	1.68	56.65	3.28	3.15
ZrS	B 11	2	3.55	6.31	1.77	79.66	5.14	5.05
ZrS_2	C 6	1	3.65	5.84	1.60	67.19	3.84	3.75
$NbS_{<1}$	WC	1	3.32	3.23	0.97	30.79	6.74	—
$NbS_{>1}$	B 8	2	3.32	6.46	1.95	61.65	6.74	—
NbS_2	C 19	3	3.33	17.80	5.35	170.7	4.58	4.40
		(rhombohedral edge = 6.24)						
		(,, angle = 30°.95)						
α-TaS_2*	C 6	1	3.35	5.86	1.75	56.80	7.16	7.10
β-TaS_2	C 27	2	3.32	12.30	3.70	117.4	6.93	
γ-TaS_2	C 19	3	3.32	18.29	5.51	174.4	7.00	6.65
		(rhombohedral edge = 6.39)						
		(,, angle = 30°.10)						
δ-TaS_2	—	6	3.34	35.94	10.75	348.5	7.02	6.85
		(rhombohedral edge = 12.14)						
		(,, angle = 15°.84)						

* At the sulfur-rich phase-limit TaS_2.

Another method of notation, which is something still more convenient in cases like the present, is to give the projections of the metal atoms on the basal plane as A, B or C and to mark the letter with a superposed – if the translation for the passage between the sulfur layers surrounding the corresponding metal layer is negative. These symbols are given in Table 6 under the heading "notation 2".

The structure of the δ-phase was already considered by HÄGG (10) but no representative seems to have been found before now.

With the assumption of close-packed sulfur lattices the stackings $(+)-$ or A and $(+)+(-)-$ or A B are the only possible ones for a hexagonal unit cell content of one and two formula units TaS_2 respectively. For γ- and δ-TaS_2 with given metal atom positions the arrangements symbolized by $(+)+(+)-(-)-$ or ACB and $(+)-(+)+(+)-(+)-(-)+(-)-$ or AACC$\bar{B}\bar{B}$ are also possible besides those given. These groupings, however, are not rhombohedral. Since h, k, l reflections with $h-k\pm l \neq 3\,n$ cannot be traced in the diffraction patterns of γ and δ-TaS_2 these arrangements are very unlikely.

It is very probable that stacking disorder of different kinds may be quite common among the TaS_2 layer lattices. The appearance of diffuse lines in some cases may be caused by such effects but generally the powder photographs show sharp lines. The four modifications described seem, therefore, to be quite well defined.

Table 8. Interatomic distances in the phases listed in Table 7

Phase	Atom	Surrounding atoms	Distances, Å	
TiS	Ti	6 S	2.49	
	S	6 Ti	2.49	
		6 S	3.30	3.52
		6 S	3.74	
TiS_2	Ti	6 S	2.42	
	S	3 Ti	2.42	
		6 S	3.39	3.42
		6 S	3.46	
ZrS	Zr	4 S	2.63	
	S	4 Zr	2.63	
		4 S	3.47	3.51
		4 S	3.55	
ZrS_2	Zr	6 S	2.56	
	S	3 Zr	2.56	
		6 S	3.60	3.62
		6 S	3.65	
Nbs	Nb	6 S	2.51	
	S	6 Nb	2.51	
		6 S	3.32	3.54
		6 S	3.75	
NbS_2	Nb	6 S	2.42	
	S	3 Nb	2.42	
		6 S	3.33	3.40
		6 S	3.46	
α-TaS_2	Ta	6 S	2.40	
	S	3 Ta	2.40	
		6 S	3.35	3.39
		6 S	3.43	
β-TaS_2	Ta	6 S	2.46	
	S	3 Ta	2.46	
		6 S	3.32	3.47
		6 S	3.62	
γ-TaS_2	Ta	6 S	2.45	
	S	3 Ta	2.45	
		6 S	3.32	3.46
		6 S	3.60	
δ-TaS_2	Ta	6 S	2.44	
	S	3 Ta	2.44	
		6 S	3.34	3.45
		6 S	3.56	

Summary

Sulfides of the transition metals Ti, Zr, Nb and Ta have been prepared in different ways and investigated by X-ray methods.

Monosulfide phases were only found to exist in the Ti-S, Zr-S and Nb-S systems. The structures of TiS and NbS are known earlier. ZrS is of the B 11 type.

No sulfide phases containing more sulfur than corresponding to the metal/sulfur ratio $^1/_2$ could be prepared. The structures of TiS_2 and ZrS_2 are known earlier. NbS_2 is of the C 19 type. Four modifications of TaS_2 of the following layer lattice types were found to exist: α (C 6), β (C 27), γ (C 19), and δ (new structure type). α-TaS_2 has a fairly large homogeneity range, approximately 5 atomic %, while the other three phases have narrow ranges. The δ-phase has probably a lower energy than the α-, β-, and γ-phases, which then are metastable. The energy of the four phases is, however, certainly very similar.

Financial support from *Statens Tekniska Forskningsråd*, which has made the present investigation possible, is hereby gratefully acknowledged.

Institute of Chemistry, University of Uppsala, May 1954.

REFERENCES

1. N. Schönberg, Acta Metallurgica 1954 (in print).
2. V. M. Goldsmidt, Geochemische Verteilungsgesetze VIII, Oslo 1927.
3. A. E. van Arkel, Physica *4*, 286. 1932.
4. W. Biltz, P Ehrlich, and K. Meisel, Z. anorg. Chem. *234*, 97. 1937.
5. E. F. Strotzer, W. Biltz, and K. Meisel, Ibid. *242*, 249. 1939.
6. N. Karlsson, J. Inst. of Metals *79*, 391, 1951.
7. N. Karlsson, Acta Chem. Scand. *6*, 1424. 1952.
8. W. Biltz and A. Köcher, Z. anorg. Chem. *237*, 369. 1938.
9. W. Biltz and A. Köcher, Ibld. *238* 81. 1938.
10. G. Hägg, Arkiv för Kemi etc. *16 B*. N:o 3. 1942.
11. F. C. Frank, Phil. Mag. Ser. 7, *62*, 1014. 1951.
12. G. S. Shdanov, C. R. Acad. Sci. U.S.S.R. *48*, 39. 1945.
13. L. S. Ramsdell, Am. Mineralogist *32*, 64. 1947.

Tryckt den 21 augusti 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 41

Communicated 14 April 1954 by ARNE FREDGA

The association of racemic and optically active α-benzyl-α-cyanopropionic acid in benzene

By SIGVARD WIDEQVIST

Carboxylic acids are to a large extent associated to double molecules in non-hydroxylic organic solvents such as benzene, chloroform, carbon tetrachloride, etc. The degree of association can easily be determined by measurements of the distribution of the carboxylic acid, at different concentrations, between the organic solvent and water. It can be assumed, *pro primo*, that no association to double molecules takes place in the water phase and, *pro secundo*, that no dissociation into ions takes place in the phase of the organic solvent.

When a monobasic acid, HA, ionizes to an extent, α, in water, the total concentrations, C_w and C_s, in the water and in the second solvent are related to each other as follows:

$$C_s/[C_w(1-\alpha)] = K_1 + 2K_1^2 C_w(1-\alpha)/K_2, \tag{1}$$

where K_1 and K_2 are the equilibrium constants, governing, respectively, the transfer of unionized monomeric molecules from the aqueous layer to the other solvent:

$$\text{HA (water)} \overset{K_1}{\rightleftharpoons} \text{HA (solvent)}$$

and the dissociation of the dimer in the non-aqueous layer:

$$(\text{HA})_2 \overset{K_2}{\rightleftharpoons} 2\,\text{HA}.$$

Thus $[\text{HA}]_s = K_1[\text{HA}]_w$ and $[\text{HA}]_s^2 = K_2[(\text{HA})_2]$, where $[\text{HA}]_w$ and $[\text{HA}]_s$ are the concentrations of the monomeric molecules in water and the other solvent, respectively. Eq. (1) was deduced by DAVIES, JONES, PATNAIK, and MOELWYN-HUGHES (1). They investigated the association of acetic acid and propionic acid in several solvents. Similar investigations have also been performed by HENDRIXON (2), MOELWYN-HUGHES (3) and HÖK (4). It should, however, be mentioned that the degree of association found in this way is dependent on the water content of the organic solvent. In dry solvents somewhat different dissociation constants are obtained as shown by VON SZYSZKOWSKI (5).

Let us now assume that we have a racemic acid the antipodes of which are denoted by HA and HB. The acid is distributed between water and benzene, and the total equilibrium concentrations in the two phases are C_w and C_s, respectively. In the same way the concentrations of the monomeric molecules are denoted $[\text{HA}]_w$, $[\text{HB}]_w$ and $[\text{HA}]_s$, $[\text{HB}]_s$. If the degree of ionization is α, we obtain for the water phase:

$$C_w = [HA]_w + [HB]_w + [A^+] + [B^+] \tag{2}$$

and

$$[HA]_w + [HB]_w = C_w(1-\alpha). \tag{3}$$

In the benzene phase we have five kinds of molecules, namely single molecules, HA and HB, and double molecules, $(HA)_2$, $(HB)_2$, and (HA, HB). The double molecules $(HA)_2$ and $(HB)_2$ must necessarily have identical dissociation constants, K_2. It is also necessary that the single molecules HA and HB have identical distribution coefficients, K_1. The dissociation constant of the complex (HA, HB) is denoted K_3. Thus we get the following relations:

$$\frac{[HA]_s^2}{[(HA)_2]} = \frac{[HB]_s^2}{[(HB)_2]} = K_2 \quad (4); \qquad \frac{[HA]_s[HB]_s}{[(HA, HB)]} = K_3 \quad (5)$$

$$[HA]_s = K_1[HA]_w \quad (6a); \qquad [HB]_s = K_1[HB]_w \quad (6b)$$

In the benzene solution we have

$$C_s = [HA]_s + [HB]_s + 2[(HA)_2] + 2[(HB)_2] + 2[(HA, HB)]. \tag{7}$$

On combining eq. (7) with eqs. (4), (5), (6) we obtain

$$C_s = K_1([HA]_w + [HB]_w) + 2K_1^2([HA]_w^2/K_2 + [HB]_w^2/K_2 + [HA]_w[HB]_w/K_3). \tag{7a}$$

But as the acid is a racemate, we must heve

$$[HA]_w = [HB]_w = \tfrac{1}{2}C_w(1-\alpha). \tag{3}$$

Thus we get from eq. (7a)

$$C_s = K_1 C_w(1-\alpha) + K_1^2(1/K_2 + 1/2K_3)C_w^2(1-\alpha)^2$$

or

$$C_s/[C_w(1-\alpha)] = K_1 + K_1^2(1/K_2 + 1/2K_3)C_w(1-\alpha). \tag{8}$$

If K_2 is known, it is thus possible to calculate K_3. The constant K_2 is determined from distribution measurements on one of the antipodes, HA or HB. It is also necessary to know the ionization constant, K_0, of the acid. The eqs. (1) and (8) are best treated by the method of least squares.

The thermodynamic ionization constant is conveniently determined from conductivity measurements by use of the $F(z)$-function of FUOSS (6).

The purpose of the present investigation was to find out if there is any relation between the racemate formation and the association in benzene of an asymmetric carboxylic acid. The measurements were performed on racemic and optically active α-benzyl-α-cyanopropionic acid. This acid has also been prepared by KENYON and ROSS (7), who started with ethyl cyanoacetate, which was methylated and benzylated. In the present investigation the starting material was α-cyanopropionic acid, prepared according to RAMBERG (8). The cyanoacid was converted to ethyl ester and benzylated. The resolution of the racemic α-benzyl-α-cyanopropionic acid was performed by means of brucine. The same method was used by KENYON and ROSS. The only difference was that whereas they used dilute acetone as solvent for the brucine salt, 50 per cent methanol was used in the present investigation.

The preparation of material

Ethyl α-benzyl-α-cyanopropionate.—In a 500-ml two-necked round bottomed flask, fitted with a reflux condenser and a dropping funnel, 11.5 g of sodium was dissolved in 170 ml of dry alcohol followed by 66 g (0.52 mole) of ethyl α-cyanopropionate. Then 65 g of benzyl chloride was added through the funnel during 20 minutes. The reaction was very vigorous. Sodium chloride separated immediately, and the heat of reaction caused the alcohol to boil. When all the benzyl chloride was added, the mixture was boiled for one hour. After cooling to room temperature, 450 ml of cold water, containing a slight amount of sulfuric acid, was added, causing the ester to separate. The ester phase was removed and the water phase extracted first with carbon tetrachloride and finally with a mixture of ether and benzene. The mixture of the separated ester and the extracts was dried with anhydrous sodium sulfate. The solvent was distilled off and the residue fractionated *in vacuo*. B.P. 161–162°/12 mm. Yield 94 g of ethyl α-benzyl-α-cyanopropionate (87 %).

Racemic α-benzyl-α-cyanopropionic acid.—Sodium, 10.5 g, was dissolved in a mixture of 200 ml of methanol and 10 ml of water, followed by 94 g of ethyl α-benzyl-α-cyanopropionate. The mixture was kept at room temperature overnight. As it still showed strong alkaline reaction, and no salt had separated, it was boiled under reflux for three hours. Dilute hydrochloric acid was then added, until the solution just showed acid reaction, after which most of the methanol was distilled off. After addition of 70 ml of water the residual methanol was distilled off. The remaining water solution was cooled to room temperature and treated with 40 ml of 12-N hydrochloric acid. The oil which separated was extracted with ether and dried over anhydrous sodium sulfate and finally over Drierite. After evaporation of the solvent the α-benzyl-α-cyanopropionic acid was obtained in crystalline state. Yield 82 g (85 %). The acid can be recrystallized from *n*-dibutyl ether or petroleum ether (B.P. 60–70°). The best solvent is, however, toluene. The acid has the melting point 95.5–96.0°. KENYON and ROSS give 93–94°.

Anal. Subs., 23.58 mg: CO_2, 60.43 mg; H_2O, 12.73 mg.
Calc. for $C_{11}H_{11}O_2N$ C, 69.81; H, 5.86.
Found C. 69.89; H, 6.04.

(+)-*α-Benzyl-α-cyanopropionic acid.*—Thirty-eight grams (0.2 mole) of α-benzyl-α-cyanopropionic acid and 93 g of brucine tetrahydrate (0.2 mole) were dissolved in 225 ml of hot methanol, after which 225 ml of hot water was added. The solution was seeded with some crystals of the brucine salt of the (+)-acid. On cooling, the salt crystallized as fine needles. Yield 50 g. The salt was then dissolved in 200 ml of hot 50 per cent methanol. On cooling, 38 g of the brucine salt separated. From the salt 10.5 g of (+)-α-benzyl-α-cyanopropionic acid was obtained. It was recrystallized from toluene. M.P. 90–91°. $[\alpha]_D^{19} = 25.3°$. KENYON and ROSS record M.P. 87.5–88.5 and $[\alpha]_D^{19} = 25.1°$.

The measurements

The thermodynamic ionization constant.—The ionization constant of α-benzyl-α-cyanopropionic acid in water at 25° was determined conductometrically. The data were treated by the method of FUOSS (6). The results are recorded in Table 1.

Table 1. Thermodynamic ionization constant of α-benzyl-α-cyanopropionic acid at 25°.

$\Lambda_0 = 371.5$

$C \cdot 10^3$	Λ_c	α	$K_0 \cdot 10^3$
1.007	316.3	0.8612	5.021
1.079	314.3	0.8562	5.124
2.152	282.3	0.7720	5.118
4.018	249.2	0.6846	5.284
4.329	243.2	0.6687	5.154
5.037	233.9	0.6438	5.132
8.660	200.6	0.5550	5.100
10.789	188.0	0.4789	5.136

Mean value $5.134 \cdot 10^{-3}$.

Table 2. The distribution of (+)-α-benzyl-α-cyanopropionic acid between water and benzene at 25°.

C_W	C_S	$C_W(1-\alpha)$	$C_S/[C_W(1-\alpha)]$
0.01483	0.02820	0.007863	3.599
1434	2652	7506	3.534
1184	1866	5843	3.193
1071	1556	5115	3.041
09113	1168	4119	2.835
08371	1000	3669	2.726
03094	01933	0866	2.232

Teble 3. The distribution of rac. α-benzyl-α-cyanopropionic acid between water and benzene at 25°.

C_W	C_S	$C_W(1-\alpha)$	$C_S/[C_W(1-\alpha)]$
0.01459	0.02760	0.007676	3.596
1407	2607	7326	3.559
1060	1547	5044	3.067
06111	05905	2367	2.495
05486	05007	2029	2.468
03173	02025	0900	2.225

The distribution measurements.—Weighed amounts of racemic and optically active α-benzyl-α-cyanopropionic acid were dissolved in analytical grade benzene and shaken with an equal volume of distilled, carbon dioxide free water in a thermostate at 25° until equilibrium was obtained. Samples were then taken from each phase with a pipette and the concentrations of acid determined by titration with 0.01911-N sodium hydroxide and phenolphthalein as indicator. For the benzene solutions a two-phase titration was employed with satisfactory results. The results of the measurements are shown in Tables 2 and 3.

Discussion

When the data of Tables 2 and 3 are treated by the method of least squares, we obtain for the (+)-acid $K_1 = 2.031$ and $2\,K_1^2/K_2 = 198.7$. For the racemic acid we get $K_1 = 2.034$ and $K_1^2(1/K_2 + 1/2\,K_3) = 205.4$. If the association to double molecules takes place to the same extent in the solutions of both the racemate and the optically active acid we should have

$$K_1^2(1/K_2 + 1/2\,K_3) = 2\,K_1^2/K_2$$

and from purely statistical reasoning we would get

$$K_2 = 2\,K_3.$$

In the present case there is in fact a small difference between the slopes $K_1^2(1/K_2 + 1/2\,K_3)$ and $2\,K_1^2/K_2$, but it is questionable if it lies beyond the limit of experimental error. The difference is in any case too small to permit any definite conclusions concerning the effect of the molecular asymmetry upon the association to double molecules in solution. The problem will, however, be the subject of further investigations.

If the mean value 2.033 is adopted for the distribution coefficient K_1, we obtain $K_2 = 0.04160$ and $K_3 = 0.01949$.

Summary

α-Benzyl-α-cyanopropionic acid has been prepared in racemic and optically active state and its thermodynamic ionization constant, K_0, determined conductometrically. By measurements of the distribution of the racemic and optically active acid between water and benzene at various concentrations the distribution coefficient, K_1, for the single molecules and the dissociation constants, K_2 and K_3, of the double molecules in the benzene phase were determined. In the benzene solution of the racemic acid the association to double molecules takes place in three ways, namely (i) between the molecules of entirely (+)-acid, (ii) between the molecules of entirely (−)-acid, and (iii) between the molecules of the (+)- and (−)-acid. The molecular complexes (i) and (ii) have identical dissociation constants, K_2, and the complex (iii) the dissociation constant K_3.

The following results were obtained:

$$K_0 = 5.134 \cdot 10^{-3} \qquad K_2 = 4.160 \cdot 10^{-2}$$
$$K_1 = 2.033 \qquad K_3 = 1.949 \cdot 10^{-2}$$

Laboratory of Organic Chemistry, University of Uppsala.

REFERENCES

1. M. Davies, P. Jones, D. Patnaik, and E. A. Moelwyn-Hughes, J. Chem. Soc. *1951*, 1249.
2. W. S. Hendrixon, Z. anorg. Ch. *13*, 73 (1897).
3. E. A. Moelwyn-Hughes, J. Chem. Soc. *1940*, 850.
4. B. Hök, Svensk Kem. Tid. *65*, 182 (1953).
5. B. von Szyszkowski, Z. physik. Chem. *131*, 175 (1928).
6. R. M. Fuoss, J. Am. Chem. Soc. *57*, 488 (1935).
7. J. Kenyon and W. A. Ross, J. Chem. Soc. *1951*, 3407.
8. L. Ramberg, Studier i kemisk kinetik, Diss. Lund 1902, p. 40.

Tryckt den 21 augusti 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 42

Read 14 April 1954

Studies on synthetic growth substances

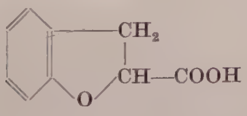
VII. The enanthiomorphs of hydrocoumarilic acid

By Arne Fredga and Carmen Vázquez de Castro y Sarmiento

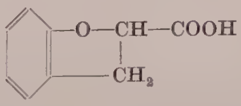
It has long been realised that substances showing auxin activity must fulfil special structural requirements (1). According to Veldstra *et al.*, the molecule must possess a basal ring system showing surface activity and a group of acid character in a certain spatial position to the ring system (2). During recent years, stereochemical aspects have attracted considerable interest (3).

Among the growth substances, both monocyclic and bicyclic ring systems are represented. For a better understanding of the growth regulating mechanism, it is of interest to investigate such bicyclic compounds, which can be formally derived from monocyclic growth substances by a ring closure, and to study the influence of this structural change on the auxin activity. Some years ago, Veldstra and van de Westeringh investigated 1.2.3.4-tetrahydro-1-naphthoic acid and compared it to α-propyl-phenylacetic acid, which can be regarded as an "open form" of the former (4). In this case the bicyclic acid was considerably more active.

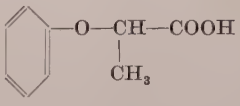
The present paper deals with the hydrocoumarilic acid (I), which has been resolved into the optical antipodes showing the m.p. 104–105° and $[\alpha]_D^{25} = +55.2°$ and $-55.0°$ respectively in benzene solution. The corresponding "open form" is the α-phenoxypropionic acid (II); if the hydrocoumarilic acid is written as Ia, the formal resemblance is more obvious.



I



Ia



II

α-Phenoxypropionic acid is an auxin of moderate activity, and according to Åberg (5), this effect is mainly due to the (+)-form, which is about 100 times more active than the (−)-form. Dr. Åberg is now investigating the enanthiomorphs of the hydrocoumarilic acid, using the S-test, and has given some preliminary results (6). The growth regulating activity is comparable to that of the α-phenoxypropionic acid, and the difference between the enanthiomorphs is of the same order of magnitude. In the case of the bicyclic acid, however, the (−)-form has the higher activity. A similar effect was observed by Veldstra and van de Westeringh (4), who found that (+)-allyl-phenylacetic acid and (−)-tetrahydronaphthoic acid are much more active than their respective enanthiomorphs. Considering what is known about the steric specificity of the auxin activity (3, 7, 8, 9), one might be tempted to assume that the compounds with higher activity are sterically related. In the present case, however, such conclusions must be presented with considerable reservation.

In the bicyclic system, the position of the atoms is much more fixed than in the "open form", where free rotation in the side chain is possible. It is doubtful, perhaps even unlikely, that the two compounds have a similar conformation, and consequently the general shape and the space requirement of the molecules may be rather different. According to ÅBERG, there is an obvious difference in the slope of the curves obtained for the acids I and II in the S-test. This may indicate a significant difference in the mechanism of the auxin action. The role of the methyl group in the α-phenoxypropionic acid (II) is also rather puzzling; the lower homologue, phenoxyacetic acid, where this group is missing, has a much weaker activity (5, 10).

The investigation will therefore be extended to other pairs of bicyclic systems and "open forms", especially those, where the question of the steric relations can be settled with synthetic methods.

Experimental

Racemic hydrocoumarilic acid was prepared according to STOERMER and KÖNIG (11). After several recrystallisations, alternately from formic acid and benzene, it melted at 116–117°.

Preliminary experiments on resolution were carried out with the usual alkaloids in different solvents (water, ethyl acetate, dilute ethanol, dilute methanol, dilute acetone). The dextrorotatory acid is best isolated through the brucine salt. Optically active benzedrine (β-phenyl-*iso*-propylamine) recommended for such purposes by MATELL (12), gives fairly good resolution; the acid obtained has the same direction of rotation as the base. (−)-Benzedrine was therefore used for the isolation of the (−)-acid.

Quinine and cinchonine give levorotatory acid of low acitivity, and cinchonidine gives excess of the (+)-form. Strychnine gives (+)- or (−)-acid according to the solvent used, α-phenyl-ethylamine acid having the same direction of rotation as the amine. The quinidine salt could only be obtained as an oil. None of these bases was suitable for the isolation of the pure enanthiomorphs.

16.4 g (0.1 mole) acid and 46.6 g (0.1 mole) brucine tetrahydrate were dissolved in the necessary amount of hot water and the salt obtained was recrystallised several times from the same solvent. The acid and the brucine can be dissolved in a rather moderate volume of water, but as the resolution proceeds, the solubility of the salt decreases very markedly. The course of the resolution was tested on samples from each crystallisation, from which the acid was liberated and examined in ethanol solution.

Crystallisation	1	2	3	4	5	6	7
g salt obtained . .	27	21	19.5	16.2	12.6	9.1	6.3
$[\alpha]_D^{25}$ of the acid . .	+14.5°	+18°	+19°	+22°	+22.5°	+22°	+22°

In a second run, 49.2 g (0.3 mole) acid and 139.8 g (0.3 mole) brucine tetrahydrate were used.

Crystallisation	1	2	3	4	5	6
g salt obtained . .	81.5	57.9	49.4	45.7	41.9	34.5
$[\alpha]_D^{25}$ of the acid . .	+13°	+19.5°	+20.5°	+21°	+21.5°	+22°

The mother liquor from the first crystallisation of the second run was evaporated to dryness and the salt obtained decomposed with dilute sulphuric acid. A hydrocoumarilic acid having $[\alpha]_D^{25} = -11.7°$ (ethanol) was obtained. 10.4 g (0.063 mole) of this acid and 8.6 g (0.063 mole) of (−)-benzedrine (prepared by recrystallisation of the neutral tartrate to constant activity) were dissolved in hot water and the salt obtained recrystallised from the same solvent. The course of the resolution was tested as described above.

Crystallisation	1	2	3	4
g salt obtained	9.9	7.3	4.9	3.1
$[\alpha]_D^{25}$ of the acid	−15.5°	−20.5°	−21°	−20.5°

As seen from the data, the maximum activity is somewhat lower than that found for the (+)-acid. A closer examination revealed that this effect was due to a slight contamination with the amine.

(+)-*Hydrocoumarilic acid.* The brucine salt (34.1 g) was decomposed with dilute sulphuric acid and the hydrocoumarilic acid isolated by extraction with ether. The yield of crude acid was 9.1 g. It was recrystallised three times from carbon tetrachloride, yielding 6.1 g of glistening needles with m.p. 104–105°. The melting point did not change further recrystallisation.

0.2211 g acid: 12.47 ml 0.1075–*N* NaOH.

$C_9H_8O_3$ Equiv. wt. calc. 164.2 found 164.9.

0.2473 g dissolved in *benzene* to 10.00 ml: $2\alpha_D^{25} = +2.73°$. $[\alpha]_D^{25} = +55.2°$; $[M]_D^{25} = +90.6°$. — 0.2584 g dissolved in absolute *ethanol* to 10.00 ml: $2\alpha_D^{25} = +1.17°$. $[\alpha]_D^{25} = +22.6°$; $[M]_D^{25} = +37.2°$. — 0.2497 g dissolved in *chloroform* to 9.99 ml: $2\alpha_D^{25} = +2.695°$. $[\alpha]_D^{25} = +54.6°$; $[M]_D^{25} = +88.6°$. — 0.2452 g dissolved in glacial *acetic acid* to 10.00 ml: $2\alpha_D^{25} = +0.665°$. $[\alpha]_D^{25} = +13.6°$; $[M]_D^{25} = +22.3°$. — 0.2648 g dissolved in *acetone* to 10.00 ml: $2\alpha_D^{25} = +0.055°$. $[\alpha]_D^{25} = +1.0°$; $[M]_D^{25} = +1.6°$. — 0.2413 g *neutralised with aqueous NaOH* and made up with *water* to 9.99 ml: $2\alpha_D^{25} = +3.695°$. $[\alpha]_D^{25} = +76.6°$; $[M]_D^{25} = +125.7°$.

Preliminary melting point determinations on mixtures of active and racemic acid indicate that the latter is a true racemate. The crystal habit is also quite different from that of the active acid.

(−)-*Hydrocoumarilic acid.* The (−)-benzedrine salt (3.1 g) was decomposed with excess of dilute sulphuric acid in the usual way and the hydrocoumarilic acid was extracted with ether. The residue obtained after drying and evaporation of the ether was, however, still contaminated with some benzedrine. It was therefore dissolved in dilute sodium hydroxide solution, and the benzedrine removed by repeated extractions with ether. The alkaline solution was then acidified with dilute sulphuric acid and the hydrocoumarilic acid isolated as usual. After drying, it was recrystallised twice from carbon tetrachloride yielding 0.95 g of glistening needles with m.p. 104–105°.

0.1904 g acid: 10.71 ml 0.1075–*N* NaOH.

$C_9H_8O_3$ Equiv. wt. calc. 164.2 found 165.3.

0.2356 g dissolved in *benzene* to 9.98 ml: $2\alpha_D^{25} = -2.595°$. $[\alpha]_D^{25} = -55.0°$; $[M]_D^{25} = -90.2°$.

Summary

Hydrocoumarilic acid (I) has been resolved into the optically active forms showing $[\alpha]_D^{25} = +55.2°$ and $-55.0°$ respectively in benzene solution. The inactive acid, melting about 12° higher than the enanthiomorphs is a true racemate.

Hydrocoumarilic acid has a weak auxin effect. This activity is mainly due to the levorotatory form.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. Börje Åberg, Royal Agricultural College, Uppsala, for kind permission to publish the preliminary reports on the growth regulating activity. Thanks are also due to AB Astra for a generous supply of benzedrine. The expenses involved in this research have been defrayed by a grant from the *Nobel Fund of the Royal Swedish Academy of Science* (*Kemiska prisgruppens särskilda fond*).

Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala, April 1954.

REFERENCES

1. Koepfli, J. B., Thimann, K. V. and Went, F. W., J. Biol. Chem. *122*, 763 (1938).
2. Veldstra, H. and Booij, H. L., Biochim. et Biophys. Acta *3*, 278 (1949).
3. Matell, M., Stereochemical studies on plant growth substances, Diss., Uppsala 1953. Also in Kgl. Lantbruks-Högsk. Ann. *20*, 205 (1953). Further references there.
4. Veldstra, H. and van de Westeringh, C., Rec. trav. chim. *70*, 1113 (1951).
5. Åberg, B., Physiol. Plantarum *5*, 305 (1952).
6. Åberg, B., Private communication.
7. Fredga, A. and Matell, M., Arkiv Kemi *3*, (nr 47) 429 (1951).
8. Fredga, A. and Westman, L., Arkiv Kemi, *7*, (nr 21) 193 (1954).
9. Mitsui, T., J. Agr. Chem. Soc. Japan *25*, 526 (1952).
10. Burström, H., Physiol. Plantarum *4*, 641 (1951).
11. Stoermer, R. and König, W., Ber. *39*, 493 (1906).
12. Matell, M., Acta Chem. Scand. *7*, 698 (1953).

Tryckt den 21 augusti 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 43

Vorgelegt am 14. April 1954

Einfluss von Tryptophan-Injektionen auf das rote Blutbild der Ratte

Von Hans von Euler, Hans Hasselquist und Erik Eriksson

Im Anschluss an Untersuchungen über chlorophylldefekte Mutanten[1] und über den Einfluss von Streptomycin[2] und dessen Antagonisten, Nukleinsäure und Purinderivate[3], auf die Chlorophyllbildung wurden auch Versuche angestellt über hemmende und fördernde Wirkungen verschiedener Stoffe auf die Bildung von Hämin. Unter den fördernden Stoffen seien besonders Glutaminsäure, Histidin und Tryptophan erwähnt, welche nach unseren[4] und nach früheren Erfahrungen als Bildner von Porphyrinen in Betracht kommen.

Im Folgenden seien über den Einfluss von Tryptophan auf den Gehalt des Rattenblutes an roten Blutzellen Versuche mitgeteilt, welche veranlasst waren durch Beobachtungen, dass in keimenden Getreidekörnern, besonders von Gerste und Weizen, in denen die Chlorophyllbildung durch Streptomycin gehemmt ist, durch Zusatz von Tryptophan bei der Quellung bzw. Keimung eine erhöhte Chlorophyllbildung eintritt, ein Effekt, über dessen Mechanismus wir uns noch nicht äussern können.

Seit langem ist bekannt, dass Tryptophan eine für das Wachstum und die Entwicklung höherer Tiere unentbehrliche Aminosäure ist. Die Grösse der Wachstumswirkung des Tryptophans ist von der Zusammensetzung der Diät der Versuchstiere im übrigen abhängig. Bemerkenswert ist in diesem Zusammenhang besonders der von Schweigert u. Mitarb. geführte Nachweis[5] der Bildung von Nikotinsäure aus Tryptophan, die nach Krehl[6] sowohl bakteriell im Darm als enzymatisch in den Organen erfolgen kann. Nach Beadle[7] entsteht Nikotinsäure aus Tryptophan wie Kynurenin aus Hydroxy-Anthranilsäure. Tryptophan beeinflusst auch den Bedarf an Nikotinsäure.

Ein Abbau des Tryptophans zu Indol, Brenztraubensäure und Ammoniak entsprechend der Synthese des Tryptophans, die aus diesen Stoffen unter der Einwirkung von Pyridoxalphosphat als Co-Enzym erfolgt, wurde kürzlich von J. Har-

---

[1] Euler u. H. Nilsson, Arkiv f. Kemi, Min. Geol. Ser. B 10, nr 6 (1929). — Übersicht über die weiteren Arbeiten über chlorophylldefekte Mutanten in: Festschrift d. Akademie d. Wissenschaften in Göttingen, Math.-physik. Klasse 123, 1951.

[2] Euler, Kem. Arb. N. F. II 9, I, 1947. — Arkiv f. Kemi, Min. Geol. Ser. A 26. nr 6, 1948.

[3] Euler u. L. Heller, Arkiv f. Kemi, Min. Geol. Ser. A, nr 14, 1948. — Euler, M. Bracco u. L. Heller, C. R. hebd. Sciences Acad. Sci. *227*, 16, 1948.

[4] Euler, Dtsch. Med. Wochenschr. *78*, 1755, 1953.

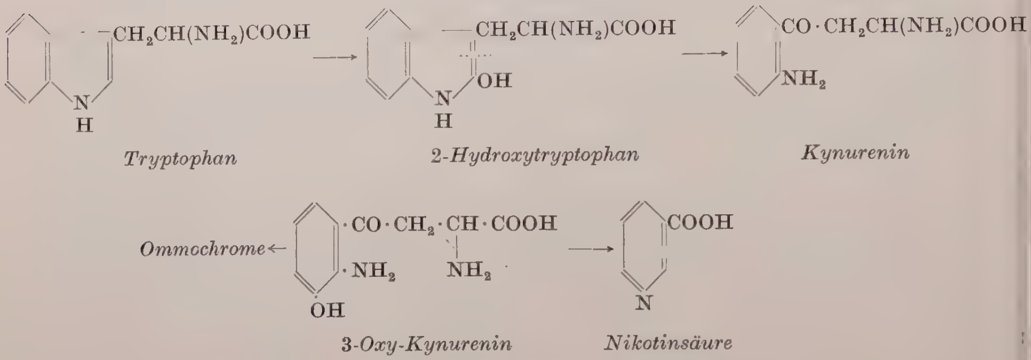
[5] B. S. Schweigert u. P. B. Pearson, J. Biol. Chem. *172*, 485, 1948.

[6] W. A. Krehl *et al.*, J. Biol. Chem. *166*, 531, 1946. — W. A. Krehl, Vit. & Horm. *7*, 111, 1949.

[7] Beadle, Proc. Nat. Acad. Sci. *33*, 155, 1947.

LEY-MASON[1] beschrieben. Wie L. M. HENDERSON und L. M. WEINSTOCK sowie M. MASON zeigten, wird bei Riboflavin- und Pyridoxinmangel in der Rattendiät der Stoffwechsel von Tryptophan verändert unter Ausscheidung von Anthranilsäure.

Tryptophan und dessen Abbaustufen haben ein besonders Interesse gewonnen durch die Untersuchungen von A. BUTENANDT[2] und Mitarb. über den enzymatischen Abbau des Kynurenins im tierischen Organismus und über den Zusammenhang des Kynurenin- und des Tryptophan-Stoffwechsels[3].



Durch Decarboxylierung entsteht aus Tryptophan das toxische Tryptamin; nach Tryptophan-Injektion in die Bauchhöhle konnten wir es nicht nachweisen.

Versuche über die Erythrozyten-Zahl des Blutes nach Tryptophan-Injektion

Tiermaterial und Methodik

Zur Verwendung kam von den beiden Rattenstämmen des Vitamin-Institutes für die vorliegenden Versuche nur unser reiner norwegischer Albino-Stamm.

Zwei Versuchsreihen wurden angestellt:

A: mit normalen, nicht vorbehandelten Tieren,
B: mit Ratten, welche gleichzeitig mit der Tryptophan-Injektion eine Injektion von Yoshida-Ascites-Sarkom erhielten.

Das Tryptophan wurde in einmaliger Dosis von 1 bezw. 2 mg, gelöst in physiologischer Kochsalzlösung, injiziert und zwar
in Serie A intramuskular (Nr. 1–3) und intraperitoneal (Nr. 5–7),
in Serie B_1 und B_2 intraperitoneal, gleichzeitig mit dem angenähert gleichen Volumen frischer Ascites-Flüssigkeit, welche unmittelbar vorher einer Ascites-Ratte nach 4–7-tägiger Inkubation entnommen wurde. Diese Tiere erhielten dadurch etwa $1 \cdot 10^6$ frische Yoshida-Ascites-Sarkomzellen eingespritzt.

---

[1] J. HARLEY-MASON, Experientia *10*, 134, 1954.

[2] A. BUTENANDT, W. WEIDEL, R. WEICHERT u. W. VON DERJUGIN, Z. physiol. Chem. *279*, 27, 1943. — A. BUTENANDT u. U. RENNER, Z. f. Naturforsch. *8 b*, 454, 1953.

[3] O. WISS, Z. physiol. Chem. *293*, 106, 1953. — O. WISS u. H. HELLMANN, Z. f. Naturforsch. *8 b*, 70, 1953.

In Serie B_2 enthielt die Tryptophanlösung gleichzeitig 0,5 mg Hämoglobin.

Nach 4–8 Versuchstagen wurden die Tiere durch Dekapitieren getötet und in dem mit ein wenig Heparin versetzten Blut wurde eine Zählung der Erythrozyten in einer Zeiss'schen Zählkammer vorgenommen. Zuweilen wurden auch die vitalfärbbaren roten Blutkörperchen (Reticulozyten) nach Färbung des Abstrichpräparates mit Brillantkresylblau gezählt.

Reticulozyten-Gehalt in normalen Ratten: Ca 1 %. Nach Tryptophan-Inj. 3 %.

In manchen Versuchen wurde das Blut der untersuchten Ratten auf den Gehalt an Brenztraubensäure (BTS) nach der Methode von Lu, modifiziert von F. Schlenk und B. Högberg, analysiert. Die BTS-Werte sind in Tumor-tragenden Tieren stark erhöht.[1, 2]

Tab. 1. Ser. A. Tryptophan-Injektionen.

Ratte Nr.	Tryptophan mg	Inj.	Inkubation Tage	Erythrozyten 10^6 per mm^3
1	3	intra-muskul.	2	9,6
2	2		3	10,1
3	1		5	11,6
Kontr.	0		0	8,8
5	2	intra perit.	3	9,3
6	2 + 2		3 (1)	9,2
7	2 + 2		3 (1)	8,9

Tab. 2. Ser. B_1 u. B_2. Injektion von Tryptophan u. Ascites-Fl.

Ratte Nr.	Tryptophan		Ascites-Fl. intraper. ml	Inkubation Tage	Erythrozyten 10^6 per mm	Tumorbildung
	mg	ml				
131	1	0,2	0,3	6	10,7	Gering; Leber hell
132	1	0,2	0,3	6	8,0	Asc. Fl. stark gelb
Kontr. 134/36	0	0,0	0,3	6	8,0	Asc. Fl. stark gelb
133	1	0,2	0,3	5	11,3	
Kontr. 134	0	0,0	0,3	5	8,0	
183	4	0,4	0,4	5		
	+ 0,5	Hämogl.				
						Grosse Milz[3]
166	2	0,2	0,3	7	8,5	Keine Gelbfärbung
167	2	0,2	0,3	6	9,2	

---

[1] Lu, Biochem. J. 33, 753, 1932. — J. Biol. Chem. 118, 427, 1937. — Papierchromatographische Scheidung des Dinitrophenylhydrazons der BTS vom Hydrazon anderer Ketosäuren: Siehe S. Markees, Biochem. J. *56*, 703, 1954.

[2] Vgl. Euler u. Mitarb. Z. Krebsforsch. *55*, 1; 1944.

[3] Ein Einfluss verschiedener an der Krebs-Entwicklung beteiligter Faktoren auf die Grösse der Milz ist wiederholt beobachtet worden.

Die Zunahme der Erythrozytenzahl durch die Tryptophan-Injektionen ist, wie die Tabellen 1 und 2 zeigen, deutlich, wenn auch klein[1]; sie beträgt bei den normalen Ratten rund 10 %, bei den Yoshida-Ascites-Sarkom-Ratten rund 20 %. Der Zusatz von Hämoglobin verstärkt die Wirkung des Tryptophans nicht (Tab. 2, Nr. 166 u. 167). Bilirubin wirkt blutbildend.

Eine Abnahme der freien Ascites-Sarkom-Zellen und des festen Sarkoms, das sich stets gleichzeitig mit den freien Zellen bildet, also eine Hemmung der Sarkom-Entwicklung durch injiziertes Tryptophan bzw. durch dessen Einfluss auf die Erythrozytenzahl, konnte nicht mit Sicherheit konstatiert werden.

In diesem Zusammenhang sei erwähnt, dass sich auch bei Stoffen, mit denen man oft grössere Hemmungen von Krebsgeweben erzielen konnte, Beziehungen zur Steigerung des oxydativen Stoffwechsels und zur gesteigerten Blutbildung zuweilen, aber *nicht regelmässig* ergeben.

Einer der wesentlichsten, wenn nicht der wesentlichste Faktor zur Förderung des normalen Stoffwechsels, durch welchen der Organismus im Kampf gegen eindringendes Krebsgewebe gestärkt wird, ist die Erhöhung der Sauerstoff-Aufnahme (Atmung) des Gewebes.

Wichtig ist nun die Frage, ob der oxydative Stoffwechsel, wie oft angenommen wird, durch Vermehrung des Cytochrom-Diaphorase-Systemes direkt gefördert werden kann. Man kann, wie früher erwähnt wurde[2], in Betracht ziehen, dass der vermehrten Cytochrom-Bildung vermehrte Porphyrin-Synthesen vorangehen müssen. Aber auch dieser Einfluss dürfte nicht hinreichen, um den gesamten oxydativen Stoffwechsel einschliesslich der damit zusammenhängenden Protein-Synthesen auf ein optimales Niveau zu heben. Vielmehr wird die allseitige Stärkung des Organismus, in welchem das normale Gleichgewicht zwischen verschiedenen energieliefernden und synthetischen Enzymreaktionen aufrecht erhalten wird, *hormonal* erfolgen müssen, ausgehend von den übergeordneten hochmolekularen Hormonen der Hypophyse.

Beobachtungen an Yoshida-Ascites-Sarkom-tragenden Ratten

Die Vermehrung des Ascites-Sarkoms nach der intraperitonealen Injektion der Ascites-Flüssigkeit geschieht vermutlich überwiegend vom sich schnell (in 3–4 Tagen) ansetzenden *festen* Sarkom aus[3], zum Teil auch durch Teilung der *freien* Sarkomzellen. Wann durch Injektion von Ascites-Tumorzellen in der Bauchhöhle nur festes Tumorgewebe und wann überwiegend *freie* Tumorzellen entstehen, hängt von noch nicht erkannten Umständen ab. Festes Sarkom wurde nach unseren Beobachtungen nie an Milz abgesetzt.

Die nach der intraperitonealen Injektion von Yoshida-Ascites-Sarkom in die Bauchhöhle unserer Ratten (sowohl unseres Albino-Stammes als des gelben Stammes BD I) auftretende Ascites-Flüssigkeit erwies sich in etwa 40 von 60 Versuchen 7–10 Tage nach der Injektion (in der Regel kurz vor dem Tod) *intensiv gelb* gefärbt. Diese gelbe Färbung trat auch sehr deutlich hervor im Eingeweide-Fett und in der inneren Hautschicht (etwa mit dem Farbton 17 des Ostwaldschen Farbenkreises).

---

[1] Bezüglich der Reticulozyten gestattet unser Versuchsmaterial noch keine Aussagen über den Tryptophan-Einfluss.

[2] Euler, Dtsch. Med. Wochenschr. *78*, 1755, 1953.

[3] Dies ist zu berücksichtigen, wenn man das Wachstum des Ascites-Sarkoms durch Zählung der freien Ascites-Zellen bestimmt.

Gleichzeitig mit der Ascites-Flüssigkeit war auch die Leber stark gelb, während dieses Organ bei schwächerer Ascites-Färbung einen grau-rosa Farbton zeigte.

Aus der gelben Ascites-Flüssigkeit kann der gelbe Farbstoff G leicht mit Benzol herausgelöst werden; er löst sich ausserdem in Chloroform, ist aber schwerlöslich in Petroleumäther und in Alkohol.

Mit diazotierter Sulfanilsäure gibt der Farbstoff G die von EHRLICH[1]-HIJMANS VAN DEN BERGH[2] für Bilirubin angegebene Rotfärbung. Geeigneter für die Kennzeichnung des Farbstoffes G ist die Fluoreszenz der mit Zinkacetat und Jod versetzten Lösung[3].

Wir haben die von mehreren Ratten gesammelten Ascites-Flüssigkeiten durch Zentrifugieren von Asciteszellen befreit und die klare, gelbe Lösung erschöpfend mit Benzol extrahiert. Die gesammelten Benzolextrakte wurden mit wasserfreiem Natriumsulfat getrocknet und im Vacuum eingeengt, bis ein gelbes Öl zurückblieb, aus dem nach eintägigem Stehen braunrote Flocken ausfielen. Durch Zusatz von Petroleumäther ging das gelbe Öl in Lösung. Die zurückbleibenden braunroten Flokken wurden mehrmals mit Petroleumäther gewaschen und hierauf in etwas Chloroform gelöst.

Einige Tropfen der stark gelben Chloroform-Lösung wurden mit 0,5 ml Alkohol verdünnt und mit 0,5 ml einer alkoholischen Zinkacetatlösung und einer Spur Jod versetzt. Nach Zusatz einiger Tropfen Ammoniak wurde Lösung im UV-Licht beobachtet. Man erhält eine für Bilirubin charakteristische Fluoreszenz. Empfindlichkeit der Reaktion 1: 12000000.

Den mittleren Bilirubin-Gehalt der gelben Ascites-Flüssigkeit fanden wir zwischen 0,3 und 0,5 ‰.[4] Auf dieser Reaktion gründeten wir eine

Papierchromatographische Methode

Munktell OB. Lösung: 8 Tle abs. Alkohol, 1 Tl Benzol.

Entwicklung: Das Papier wurde mit alkoholischer Zinkacetatlösung bespritzt, die eine Spur Jod enthielt. Während 1–2 Minuten liess man Ammoniakdämpfe auf das Papier einwirken. Im UV-Licht treten dann die Flecken mit roter bis grüner Fluoreszenz hervor.

Vergleich mit einem reinen Bilirubin-Präparat: Sowohl dieses wie unser gereinigter Farbstoff G zeigten ganz übereinstimmende R_f-Werte 0,83–0,85. Die Chloroformlösung der gelben Ascites-Flüssigkeit enthielt *ausserdem noch einen zweiten gelben Farbstoff* von $R_f = 1$, der aber im UV-Licht keine Fluoreszenz zeigte.

Versuche mit gealterter Ascites-Flüssigkeit

Ratten Nr. 106 und 107.

Zum erstenmal injiziert den 25.II. 54 mit Ascites-Flüssigkeit (Asc. Fl.), welche 28 Stunden bei 0° verwahrt worden war. Den 9.III. (also nach 12 Tagen) zweite Injektion mit frischer Asc. Fl.

---

[1] EHRLICH, Zbt. f. klin. Med. *4*, 721, 1883. — Verwendung von diazotiertem *Dichloranilin* vgl. P. SACHS, Zs. klin. Med. *119*, 551, 1932. — Z. physiol. Chem. *242*, 19, 1936.

[2] HIJMANS VAN DEN BERGH u. MÜLLER, Biochem. Zs. *77*, 90, 1916: 1936.

[3] Siehe HANS FISCHER u. HANS ORTH, Chemie des Pyrrols, II. Band, 1. Hälfte, S. 632, 1937.

[4] Die Ratten entbehren bekanntlich eine Gallenblase. Der Austritt des Gallenfarbstoffes aus den Gallengängen der Leber erfolgt vermutlich an den mit Tumorzellen besetzten Stellen, besonders an der Verbindungsstelle der vier Leberlappen.

Nr. 106. Nach 7 Tagen verendete das Tier mit ganz normal entwickelter Asc. Fl. und normal entwickeltem Tumor.

Nr. 107. Erst am 24.III (nach 12 Tagen) nussgrosser Tumor and der Injektionsstelle. Am 27.III. anscheinend nicht leidend; dekapitiert. Keine Spur von Asc. Fl. Ausser dem schon erwähnten nussgrossen Tumor ein weiterer grosser Tumor in der unteren Bauchhöhle sowie ein haselnussgrosser Tumor zwischen Magen und Leber, vom Aussehen eines Jensen-Sarkoms. Gesamtgewicht der Tumoren 28 g.

Ratten Nr. 159, 160 und 161.

Injektion am 19.III. 54 mit 4 ml Asc. Fl., die bei 0° 4 Tage lang aufbewahrt worden war. Gewichtszunahme in 12 Tagen 19 g. Den 31.III. wurden die gleichen Ratten zum zweitenmal, und zwar nun mit *frischer* Asc. Fl. injiziert.

Nr. 161. Am 7.IV. 54 sichtlich leidend, aber Bauch kaum geschwollen. Dekapitiert. Reichliche Bildung von festem Tumor; aber nur 1 ml Asc. Fl. Der Brusthöhle konnten 2 ml mit Blut vermischte Flüssigkeit entnommen werden. Am Thymus eine grosse, rote Masse, anscheinend ein Ascites-Tumor.

Nr. 160. Am 13.IV. dekapitiert. Man fand 2 ml grau-rosa Asc. Fl. Grosser, fester Tumor; ausserdem mehrere kleine Tumoren in der Bauchhöhle. 1 Lebertumor von Erbsengrösse. Gesamtgewicht der Tumoren 16,5 g.

Nr. 159. Den 12.IV. sichtlich leidend; grau-rosa Asc. Fl. mit sehr wenig festem Ascites Tumor. In der Bauchhöhle ein 6,5 g Tumor vom Aussehen eines Jensen-Sarkoms. Ausserdem mehrere ähnliche Tumoren in der Bauchhöhle. Milz 3,7 g. — Leber 18,3 g.

Von den 5 untersuchten Ratten (106, 107; 159, 160 und 161), welche mit gealterter Asc. Fl. injiziert worden waren, trat nur in *einem* Fall bei erneuter Injektion ein normaler Ascites-Tumor auf. In den 4 anderen Fällen traten nach geringen Mengen Asc. Fl. grosse, feste Tumoren auf, deren Aussehen von demjenigen der Ascites-Tumoren wesentlich abwich.

Eine erworbene Resistenz bzw. Immunität gegen den Ascites-Tumor konnten wir nie beobachten. Es mag dies damit zusammenhängen, dass in den gealterten Asc. Fl. wenig lebende bzw. virulente Zellen vorhanden sind; auf die in solchen Zellen noch vorhandenen Ergone werden wir bald zurückkommen.

Röntgen-bestrahlte Ascites-Flüssigkeit aus Ratten 176 und 177

Ratte Nr. 176.

Die am 29.III. mit 500 r bestrahlte Ascites-Flüssigkeit, welche noch einen Teil ihrer Virulenz besass, wurde eine Stunde nach der Bestrahlung injiziert. Nach 5 Tagen wurde das injizierte Tier dekapitiert. In demselben fand sich nur ein ganz kleiner Tumor, etwa von der Grösse eines Reiskorns.

Ratte Nr. 177.

12 Tage nach der Injektion der bestrahlten Ascites-Flüssigkeit dekapitiert. Wenig aber stark gelbe Asc. Fl. Fester Tumor an der Injektions-Stelle. Reichlich freie Ascites-Sarkomzellen. Leber angegriffen. Die Ascites-Fl. von Ratte 177 wurde in Ratten 203 und 204 injiziert. Entwickelte am 14.IV. noch keinen Tumor.

Anhang. Versuche mit Thymusnukleinsäure

Im Anschluss an die in Tab. 1 und 2, S. 3, mitgeteilten Versuche über den Einfluss des Tryptophans auf die Entwicklung des Yoshida-Ascites-Sarkoms und auf die in den betr. Ratten bestehende Erythrozytenzahl wurde auch Desoxyribonukleinsäure[1] in die Yoshida-Ascites-Sarkomratten intraperitoneal eingespritzt. Dose 0,5 mg.

Starke Veränderungen der Tumorentwicklung und der Erythrozytenzahl haben sich dadurch nicht ergeben.

---

[1] Darstellung: L. AHLSTRÖM, EULER, I. FISCHER, L. HAHN u. B. HÖGBERG, Arkiv f. Kemi, etc. 20 A, nr 15, 1945.

Stockholm, Universität, Vitamin-Institut und Inst. für organ.-chem. Forschung.

Tryckt den 21 augusti 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 44

Communicated 14 April 1954 by PER ERIC LINDAHL

Incorporation of S^{35} in sperm-antagglutin

By J.-E. KIHLSTRÖM

With 1 figure in the text

In 1952 LINDAHL and KIHLSTRÖM demonstrated the occurrence of an antiagglutinic effect in semen from bull, swine and rabbit, and isolated in a crude form a non species-specific antiagglutinic factor from bull semen. Later on this substance, named sperm-antagglutin, was purified and shown to be an acid proteid, containing, *inter alia*, carbohydrate (LINDAHL and KIHLSTRÖM, 1954). From this complex proteid a non-proteinic active group, having the same properties as antagglutin itself, can be split off. The biological activity is restricted to the reduced form of antagglutin, which adheres to the surface of the spermatozoa and is released upon oxidization.

The reduced form of sperm-antagglutin as well as of its active group shows metachromatic staining with Azur A. According to modern views metachromasia is not specific to sulphuric esters of high molecular polysaccharides, as was formerly believed, but is probably due to a certain density of negatively charged groups. (BANK and BUNGENBERG DE YONG, 1939; WIAME, 1947). However, the low isoelectric point (2.2 and 3.0 for oxidized and reduced forms respectively) of antagglutin suggests the occurrence of strongly acid groups. As phosphate is absent, it seems probable that sulphate groups are responsible for the acid character of the antagglutin.

The small quantities of antagglutin available up to the present day made it difficult to demonstrate the presence of sulphate by chemical methods. For this reason it appeared very interesting to investigate if S^{35} is incorporated into antagglutin.

Methods

Three male rabbits were given $Na_2S^{35}O_4$ intraperitoneally 1, 2 and 3 times respectively, each dose containing 0.7 mC labelled sulphur. Semen was collected daily from the rabbits by means of an artificial vagina, and the sperm cells centrifuged down (9,000 g 10 min.). 0.05 ml of the clear sperm plasm was evaporated and the radioactivity determined in a Geiger-Müller counter. From the rest of the sperm plasm antagglutin was prepared according to LINDAHL and KIHLSTRÖM (1954). In order to remove inorganic sulphate the antagglutin solutions were carefully electrodialyzed against distilled water. After evaporation the radioactivity of the dry substance was measured. The amount of antagglutin in a given preparation was determined by extinction measurement at 270 mμ. The relation between extinction and dry weight of antagglutin solutions was found to be 1.59 mg/ml/extinction unit.

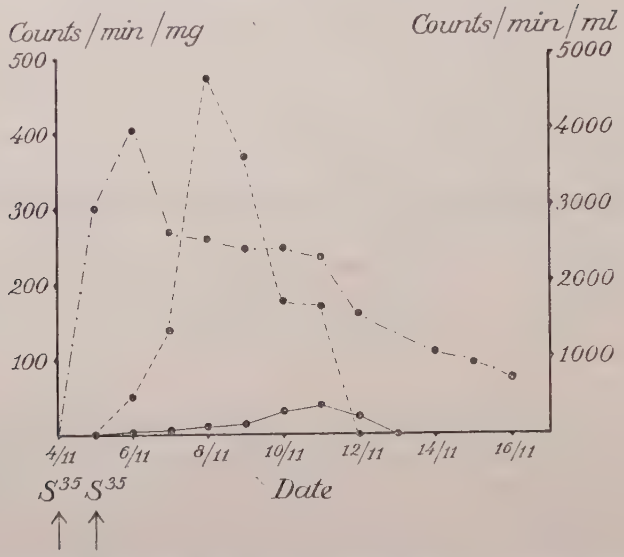


Fig. 1. The radioactivity, registered by a Geiger-Müller counter, of sperm plasm (● — · — ●), isolated antagglutin (● — — — ●), and the remaining proteins (●———●) after injection of S^{35}-labelled sodium sulphate into a rabbit.

The values obtained for bull (LINDAHL and KIHLSTRÖM, 1954) and rabbit antagglutin are of the same order of magnitude.

The sperm plasm freed from antagglutin was saturated with ammonium sulphate and the protein precipitate centrifuged down (9,000 g 10 min.). The sediment obtained was dissolved in distilled water and the salting out procedure repeated. A solution of this second sediment was electrodialyzed against distilled water and evaporated. The amount of protein per sample was determined by weighing on a microbalance, and the radioactivity measured.

The radioactivity is calculated as counts per min. per mg protein and, in the case of seminal plasma, as counts per min. per ml. The half-life period of the isotope used being known, all figures refer to the date of the beginning of the experiments. The number of counts measured exceeded in each sample 1,000, giving 2 % statistical uncertainty (HEVESY, 1948). The background of the counter was measured with the same accuracy.

Results

For obvious reasons the experiments are not absolutely identical. However, the general trend is the same and the results shown in Fig. 1 may be taken as representative.

For a few days after the first injection no labelled sulphur can be detected in the proteins, but the sperm plasm shows high radioactivity, which may derive from inorganic sulphate in the plasm. Two or three days after the first injection some S^{35} has been incorporated into the proteins. However, antagglutin shows a more rapid incorporation than the other proteins. Four days after the first injection maximal amounts (in Fig. 1 corresponding to 470 counts/min./mg protein) of the labelled sulphur are found in antagglutin. The other proteins, regarded as a unit, show the corresponding maximum (40 counts/min./mg protein) two days later. Nine to ten days after the first injection no labelled sulphur is detectable in the proteins. The sperm plasm, however, still contains radioactive substances, most probably organic compounds other than proteins.

Discussion

These experiments demonstrate the occurrence of sulphur in sperm-antagglutin, but they tell nothing about the chemical nature of the former.

Boström (1953) found that in rats neither cysteine nor methionine shows any significant incorporation of S^{35}. According to these findings the labelled sulphur should not be expected to be incorporated into the protein residue of the antagglutin molecule. Boström (loc. cit.) also found a low, but significant, incorporation of S^{35} into taurine. As cysteine is generally believed to be the precursor of taurine, there is some contradiction here and the possibility of incorporation of labelled sulphur into the protein part of the molecule cannot be excluded altogether. It thus seems most probable that the main part of the labelled sulphur administered is incorporated into the active group of antagglutin and not into the protein residue.

The low isoelectric point of antagglutin suggests the sulphur to exist mainly in the form of sulphate.

ACKNOWLEDGMENT

I am very much indebted to Professor P. E. Lindahl for advice and valuable discussions.

Institute of Zoophysiology, University of Uppsala, Sweden.

REFERENCES

Bank, O. and Bungenberg de Young, H. G., Protoplasma *32*, 489, 1939.
Boström, H., Arkiv för kemi, *Band 6*, Nr 5, 43, 1953.
Hevesy, G., Radioactive Indicators. New York 1948.
Lindahl, P. E. and Kihlström, J.-E., Report of the II International Congress of Physiology and Pathology of Animal Reproduction and of Artificial Insemination, 70, 1952.
—— Fertility and Sterility, 1954. (In press).
Wiame, J. M., Am. J. Chem. Soc. *69*, 3146, 1947.

Tryckt den 21 augusti 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 45

Read 28 April 1954

Starch degradation by salivary amylase

III. Residual dextrins and the glucose formation

By KARL MYRBÄCK and EBBA WILLSTAEDT

With 2 figures in the text

The incomplete saccharification of starch and glycogen by the amylases, known since the beginning of starch chemistry, is explained by the fact that together with the chief products of the degradation, i.e., the fermentable sugars, maltose and, at least sometimes, glucose and maltotriose, there are formed in the amylase reaction with amylopectin and glycogen rather large quantities of non-fermentable saccharides having a lower reducing power and, consequently, a higher degree of polymerization (DP) than maltose. These saccharides, which are not or in any case only very slowly, attacked by the amylases, have been termed residual dextrins (stable dextrins, limit dextrins). Investigations on these residual dextrins after degradation of starch by α-amylases (cf. Ref. 1) and removal of the sugars by yeast fermentation have revealed that the residual dextrins actually isolated are always mixtures of several saccharides of varying DP. The average DP generally seems to be somewhere around 5. Tetra-, penta-, and hexasaccharides are often found in abundance, sometimes also trisaccharides, In a few cases, especially after starch degradation with crude mold enzymes, a non-fermentable disaccharide fraction could be isolated. Small amounts of residual dextrins with DP > 6 are very often present.

It was suggested by one of us in 1937 (2) that the starch residual dextrins are primary products of the enzyme action, i.e., fragments of the original substrate molecules, and that these fragments are (more or less) stable against enzymatic attack because they are structurally different from the main part of the substrate chain-molecules which is transformed into fermentable sugars by the amylases. Accordingly it was concluded that the sole action of the ordinary amylases is the hydrolysis of 1,4-α-glucosidic linkages, and that in starch or a starch component (amylopectin), and in glycogen, structural "anomalies" occur, i.e., deviations from the simple starch formula advocated by HAWORTH. The anomalies were supposed to be glucosidic linkages other than the normal 1,4-α-linkage (with or without ramification of the chains), substitution of glucose units by phosphoric acid, etc. It is clear by now that the anomalies chiefly responsible for the residual dextrin formation are the 1,6-α-glucosidic linkages in amylopectin and glycogen, while other anomalies such as the substitution with phosphoric acid are by far less important. The occurrence in a residual dextrin trisaccharide of one 1,6-α-glucosidic linkage was demonstrated (3) and also the formation of isomaltose from starch by the action of takadiastase (4). The general occurrence of at least one isomaltose linkage in every residual dextrin molecule was made probable (5, 3). It seems that these ideas have been generally accepted.

Pringsheim (6) had the opinion that not only the residual dextrins but also maltose are secondary products, formed by synthesis from glucose that was thought to be the one and only real primary product of amylase action. It was not difficult to show that this theory is untenable. However, it must be conceded that recent results concerning transglucosylating enzymes may also have a bearing on the question of the residual dextrins. Especially with crude mold enzyme preparations it can not be excluded that saccharides similar to, or even identical with, certain residual dextrins may arise by transglucosylation, e.g. from isomaltose. However, many amylase preparations, such as salivary amylase (7), do not contain transglucosylating enzymes. The fact that the yield and average composition of the residual dextrins produced by different α-amylases do not vary strongly seems to indicate that transglucosylations generally do not play any important role in the residual dextrin formation. On the other hand, all α-amylases must not necessarily be thought to produce the identical residual dextrins.

Meyer *et al.* (8) have made the over-simplified statement that all α-amylases degrade amylopectin completely into maltose, glucose and isomaltose, the latter of which is regarded by these authors as the only true limit dextrin. Their statement was based upon determinations of reducing power and fermentation experiments on a small scale, not very well suited for the elucidation of the nature of the real end-products of the α-amylase degradation. The only known case in which the final composition of the degradation products may correspond approximately to the Meyer theory is that of starch degradation by crude mold amylase preparations. However, these preparations are known to contain a variety of carbohydrases, including "limit dextrinase", maltase, etc. Actually, such preparations may yield glucose and isomaltose as the chief end-products (9); other preparations may also hydrolyze isomaltose and thus effect a complete degradation of amylopectin to glucose (10). However, as mentioned above, it is a well-established fact that most α-amylase preparations acting on the branched substrates yield considerable amounts (from starch 15–20 per cent) of residual dextrins, of which the average DP is 4–5. Since starch contains *c.* 4 per cent non-reducing end-groups, i.e. *c.* 4 per cent 1,6-α-glucosidic linkages, the yields and average DP of the residual dextrins are in agreement with the assumption that, in general, every residual dextrin molecule contains one 1,6-α-glucosidic linkage (1, 3, 5).

In the case of saliva preparations the danger of contamination of the amylase with other carbohydrases appears to be smaller than with other α-amylases. Salivary amylase is an extremely stable enzyme and can be purified considerably and also obtained in crystalline form without too much difficulty. The enzyme should be well suited for experiments on the nature of the end-products of its action on starch and other substrates. Using highly purified enzyme in very large amounts we found (11) that even after extremely prolonged action of the enzyme at least 13 per cent (calculated on the amount of glucose theoretically obtainable from starch) remained as non-fermentable residual dextrins with DP ~ 5. The action of the same enzyme on isolated fractions of these dextrins was so weak that its nature and origin appeared doubtful.

In a later paper (12) similar experiments were reported. The residual dextrins were isolated and roughly separated into several fractions by ethanol precipitation.

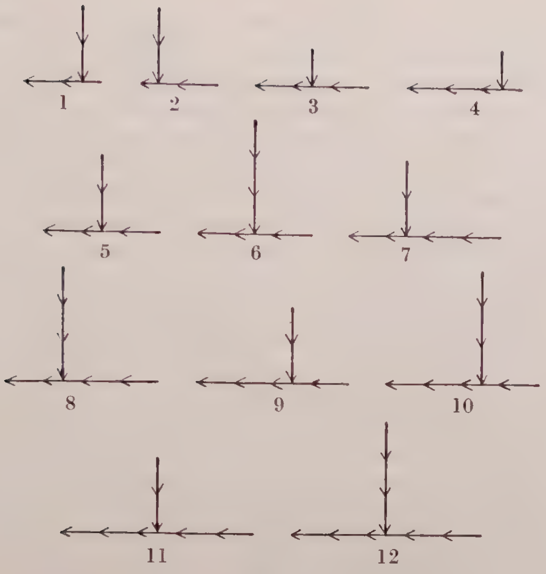


Fig. 1.

Each fraction was investigated by paper chromatography. No isomaltose was found and only small traces of trisaccharides. Saccharides with 4, 5, 6, 7, and sometimes more, glucose units were the components. Treatment of the fractions with large amounts of salivary amylase resulted in an extremely slow and incomplete secondary hydrolysis. Fermentable sugars were produced and the amount of tetrasaccharide increased, whereas trisaccharides and isomaltose were absent. It was suggested, therefore, that a tetrasaccharide, containing one 1,6-α-glucosidic linkage, may be regarded as the true limit dextrin of salivary amylase or at least one of the true limit dextrins. The structure of the tetrasaccharide would be represented by one of the formulas 1–4 in Fig. 1.

In a following paper (13) experiments were reported which may indicate that the tetrasaccharide limit dextrin is 4-α-panosido-D-glucose (formula 4 in Fig. 1). However, since the R_F- or R_G-values, respectively (14), of the possible isomers, are not known, the evidence for this structure is not conclusive.

Important contributions to the knowledge of enzymatic starch degradation have been made by WHELAN *et al.* (15, 16). While our results are, on the whole, in good agreement with those of the cited authors, there are certain differences of opinion. WHELAN and ROBERTS (16) believe that the lowest residual dextrin of salivary amylase is a pentasaccharide having the structure shown in formula 5, Fig. 1. This structure would imply that salivary amylase is unable to split 1,6-linkages and the three 1,4-linkages of a ramified molecule of the amylopectin type, adjacent to the ramification point (1,6-linkage). Since the authors suppose that the enzyme is also unable to rupture terminal 1,4 linkages, they assume that salivary amylase will produce the limit dextrins Nos. 5–12 in Fig. 1. Furthermore, they assume, as also done by us (14), that residual dextrins containing more than one 1,6-linkage per molecule may occur in small amounts. This would be the case especially with glycogen on account of the low average length of the interior chains in this substrate.

The evidence presented by WHELAN and ROBERTS for the assumed structure of the pentasaccharide (No. 5 in Fig. 1) seems convincing. On the other hand we are satisfied that the lowest residual dextrin present in our preparations is a tetrasaccharide; in several of our dextrin fractions the tetrasaccharide was actually the main constituent. We believe that the discrepancy between these results and those of WHELAN and ROBERTS, who could find no residual dextrin lower than the pentasaccharide, is only apparent and dependent on the intensity of the enzyme action on the substrate (enzyme concentration and reaction time), and possibly also on the purity or enzymatic homogeneity of the enzyme preparations used. It is well known that when α-amylases act on starch or glycogen, the reaction rate, after an initial period of rapid hydrolysis, gradually decreases without actually reaching zero in a reasonable time. As we have pointed out before, it is often practically impossible to decide when the "end-point" of the reaction, the "saccharification limit", has been reached. In any case it is practically impossible to state that a definite end-point has been reached solely on the basis of the reducing power of a reaction mixture. In other words, it is difficult to decide if isolated or otherwise characterized degradation products are really "end-products". Of course, it is always possible to isolate a product and to find out, in suitably arranged experiments, if the product is stable or not against the enzyme used in the preparation of the product. However, if, in such experiments, the product in question turns out to be attacked by the enzyme and the rate of the resulting hydrolysis is very small compared to the action of the same amount of enzyme on starch, then the difficulty is to decide if the observed slight action is really an action of the amylase proper and not an action of some contaminating enzyme (e.g., a "limit dextrinase"). It certainly is a truism that this question can be solved by using a pure or at least an enzymatically homogeneous enzyme preparation. It is also true that salivary amylase can be purified fairly easily and obtained in crystalline form. However, in the case of proteins the crystalline state is by no means proof of homogeneity. As we have found in several cases, a secondary hydrolysis of residual dextrins by highly purified salivary amylase may well proceed at a rate less than 1/10,000 of that of the same enzyme on starch (11). Therefore, if this hydrolysis of a residual dextrin is caused by a contaminating carbohydrase, the amount of this enzyme in the amylase preparation may be less than 0.01 per cent. Obviously, an assertion that the enzyme is "pure" in the conventional interpretation of the word has no real meaning in a situation like this.

How difficult the problem is can be exemplified by quoting the results of different authors on the action of salivary amylase on maltotriose: MYRBÄCK and LEISSNER (17) concluded that maltotriose is stable against salivary amylase. Since the experiments were performed with normal enzyme concentrations and fairly normal reaction time the result would be that, if maltotriose is hydrolyzed by salivary amylase, the rate of this hydrolysis must be less than about 1/100 of that of starch hydrolysis. MEYER *et al.* (8), as already mentioned, concluded from their experiments that salivary amylase (and other α-amylases) hydrolyze maltotriose to form maltose and and glucose. ROBERTS and WHELAND (15) again found maltotriose to be stable, whereas MEYER and GONON (8), using crystalline enzyme, found hydrolysis to glucose and maltose. HOPKINS and BIRD (18) conclude that in a saccharide chain the terminal linkage at the reducing end and two linkages at the non-reducing end are non-accessible or at least difficultly accessible to salivary amylase. In our later experiments with salivary amylase preparations of varying degree of purity we always found initially the formation of considerable amounts of maltotriose which, however, then generally decreased and sometimes vanished completely after long periods of time. This was also the case with enzyme preparations purified according to the methods of MEYER *et al.* (crystallization stage). Recently PAZUR (19) has reported experiments on the action of salivary amylase on certain low-molecular saccharides. The author obviously does not question the statement of MEYER that human saliva contains only one amylase and no other enzymes, and he therefore uses saliva without any pretreatment. He finds that the enzyme has no action on maltose (0.2 ml saliva, 50 mg maltose in 1 ml phosphate buffer of pH = 6.8, 200 hrs.), but maltotriose is split slowly into maltose and glucose. Maltotetraose was split rapidly and the reaction products were chiefly maltose but also some glucose. This is taken to show that the central linkage of maltotetraose is ruptured rapidly (yielding two molecules of maltose), and one of the terminal linkages comparatively slowly, yielding glucose and maltotriose.

Evidence has been presented from this laboratory to show that the glucose formation by α-amylases, and especially malt α-amylase, takes place by the liberation of glucose units from the reducing end of saccharide chain-molecules (20, 21). On the other hand, nothing is known that could support an assumption that glucose can be split off by the amylases from a non-reducing chain-end. If maltotriose and a terminal linkage of maltotetraose are split by salivary amylase it therefore seems permissible to assume that the linkage ruptured by the enzyme is that adjacent to the reducing end-group.

The simplest explanation of the conflicting results quoted above seems to be that human saliva, contrary to the statement of MEYER (8), contains not only the true α-amylase but also some other enzyme capable of producing glucose from short-chain degradation products, e.g., maltotriose. The same must be said concerning the ability of salivary preparations to hydrolyze maltose. As we have already reported (22) and as will be seen in the following, our preparations have a definite action on maltose. This may be because of the presence of a maltase in our saliva samples, but in our experiments even very highly purified salivary amylase had an action on maltose. It does not seem quite impossible to assume that, if salivary amylase has a more or less slow action on the terminal linkage adjacent to the reducing group of maltotetraose and maltotriose, it could also have an action, probably still slower, on the linkage of maltose. Or, it could be that the amylase is contaminated with an enzyme other than maltase, capable of producing glucose from short-chain saccharides including maltose.

In view of the conflicting results quoted it does not seem easy to arrive at a solution of the problem. The only way appears to be to compare the action of different salivary amylase preparations, not necessarily of the highest amylatic power, but treated by various means calculated to remove contaminating enzymes or to differentiate between enzymes, e.g., by partial heat inactivation or by treatment with sorbents.

In the following, some experiments with partially heat-inactivated saliva are described.

Experimental

Substrates. 1, Maltose, commercial product; 2, Lintner starch, prepared from native potato starch (Lintner starch No. I: 1, cf. (23)); 3, residual dextrin fraction S I; 4, residual dextrin fractions by hydrolysis of potato starch with salivary amylase and ethanol precipitation has been described briefly (13): Treatment of 15 l about 5.6 per cent starch paste with 175 ml saliva at 45° during

Table 1. Residual dextrin fractions

Dextrin fraction No.	Yield, per cent of starch	Average DP
S I	6.3	7.0
S II	2.8	6.5
S III	1.9	6.3
S IV	1.3	5.5
S V	0.9	5.1
S VI	0.5	3.8
S VII	0.2	2
Total yield 13.9 per cent	DP (weighted average) 6.35	

20 days yielded about 18 per cent non-fermentable dextrins containing 4–7 glucose units together with smaller amounts of higher dextrins. The average DP of the dextrins was 5.7. Repeated treatment of these dextrins with large amounts of enzyme (150 ml saliva in 850 ml total volume) caused a slow and incomplete saccharification that appeared to have stopped after 17 days at 45°. The residual dextrins were isolated after removal of the fermentable sugars with baker's yeast and roughly fractionated by alcohol precipitation: Table 1.

Paper chromatographical examination showed that all fractions except the small fraction S VII (probably rather impure) contained saccharides with 4 and more glucose units. In fraction S VI the tetrasaccharide(s) predominate strongly in accordance with the average DP = 3.8. Fraction S V contains tetra-, penta-, and hexasaccharides, and possibly traces of heptasaccharides. Fractions S I–S IV contain saccharides with 4–7 units, fractions I and II also saccharides with 8 or more units. It was suggested that the dextrins with more than 7 units have two or possibly more 1,6-linkages. In the dextrin fractions, S I–S III, the hexasaccharides appear to be the chief components.

Enzyme. Filtered human saliva, stored under toluene. Portions of the saliva were heated at pH = 6.5 and 63°, 64°, and 65°, respectively: Thin-walled tubes containing the saliva were immersed in a waterbath at the desired temperature; after 15 minutes the tubes were cooled in tap water. At + 3° under toluene the saliva solutions appeared to retain the activity indefinitely.

Activity of the enzyme preparations. 25 ml 2 per cent Lintner starch solution, 19 ml water, 5 ml phosphate buffer of pH = 6.9 and 0.1 g NaCl were mixed and temperated at 30°. 1 ml enzyme solution was added and the increase of the reducing power was determined in small samples according to the Willstätter and Schudel method: Table 2.

Table 2. Action of saliva preparations on starch

Reaction time, min.	Reducing power, calculated as per cent glucose			
	Enzyme not heated	Enzyme heated to		
		63°	64°	65°
2	9.3	2.6	—	—
4	15.8	7.3	7.2	3.2
8	23.8	12.8	11.0	5.8
12	26.6	18.4	15.6	7.3
20	—	23.1	21.6	12.5
50	—	—	—	20.5
Activity	5.25	2.20	1.6	0.73
Relative activity, per cent	100	42	30	13

If the initial velocity of the starch hydrolysis is estimated graphically from the hydrolysis curves corresponding to Table 2 and is expressed as the relative number (per cent) of substrate linkages ruptured per minute, the activity values recorded in the bottom lines of Table 2 are obtained.

Action of saliva preparations on maltose

The following mixtures were prepared and stored under toluene at 40 ± 1° in a temperature-regulated room: 2 g maltose hydrate, 0.1 g NaCl, 2 ml phosphate buffer of pH = 6.9, 93 ml water and 5 ml saliva preparation. After suitable intervals paper chromatograms were prepared as described earlier (14), and the formation of glucose was determined by fermentation experiments (24). Results in Table 3; the number of crosses gives a rough idea of the intensity of the glucose spots.

It is seen from Table 3 that the liberation of reducing groups from maltose is about 0.5 per cent per day for 5 ml unheated saliva in 100 ml 2 per cent maltose solution at 40°. The rate of starch hydrolysis by the same enzyme (Table 2) is 5.25 per cent per minute for 2 ml enzyme in 100 ml 1 per cent starch solution at 30°. If the reaction rate at 40° is supposed to be twice that at 30°, and if we disregard the difference in substrate concentration which does not play any too important role, we find the ratio between the two reaction velocities to be approximately 1/75,000. In the case of the heated saliva preparations the experiments with maltose are not accurate enough to permit a similar calculation, but all three solutions have a distinct action on maltose. Its order of magnitude seems to be about 30 per cent of that of the unheated solution. The partial heat inactivation of the amylase solution has evidently not brought about any great alteration of the ratio of the two hydrolysis rates. The experiments do certainly not prove that the activity of the saliva against maltose is a property of the pure salivary amylase; on the other hand, since salivary amylase is a remarkably stable enzyme while the known maltases are generally very unstable in solution, one may be tempted to ask if the observed action of saliva on maltose is not an action of salivary amylase.

In a previous paper (22) we have reported that heated preparations of salivary amylase, which had lost most (but not all) of their activity against maltose, still formed glucose from starch or its degradation products. Considerably more glucose was formed from starch than from maltose and, consequently, terminal linkages in higher saccharides must, in any case, be ruptured with greater ease than the linkage of maltose.

Table 3. Action of saliva preparations on maltose

Days	Enzyme not heated		Enzyme heated to					
			63°		64°		65°	
	Glucose spots	Per cent hydro-lysis	Glucose spots	Per cent hydro-lysis	Glucose spots	Per cent hydro-lysis	Glucose spots	Per cent hydro-lysis
3	+ +	2	+	?	+	?	+	?
15	+ + +	8	+	2	+	2	+	2
53	+ + + +	15	+ +	5	+ +	5	+ +	4
250	—	—	+ + + +	—	+ + + +	—	+ + + +	*

* Spot intensity indicated a conversion of about 30 per cent.

Action of saliva preparations on limit dextrin fraction S I

The following mixtures were kept under toluene at 40°: 4 g (dry weight) dextrin, 0.2 g NaCl, 4 ml phosphate buffer of pH 6.9, 186 ml water and 10 ml saliva. The increase of the reducing power was determined in small samples and calculated as per cent glucose, Table 4. Paper chromatograms were prepared and examined as previously described.

Table 4. Action of saliva preparations on fraction S I

Days	Reducing power, per cent glucose			
	Enzyme not heated	Enzyme heated to		
		63°	64°	65°
0	16.0	16.0	16.0	16.0
3	—	16.2	—	16.2
14	16.8	16.2	—	16.1
25	17.7	—	—	—
53	19.6	17.0	16.9	16.1
250	20.6	17.3	17.3	16.5
Fermentable sugar, per cent	~8	~4	~4	~4

The yield of fermentable sugar was so low that the figures of the bottom line of Table 4 are rather uncertain. However, both fermentation experiments and the chromatographic determinations showed the presence of glucose and also small amounts of maltose. The spots on the papergrams corresponding to tetrasaccharide limit dextrin ($R_G = 0.264$) were somewhat stronger after the amylase treatment, the heptasaccharide spots ($R_G = 0.070$) perhaps somewhat weaker. No formation of trisaccharides or isomaltose could be observed.

Since the enzyme action in these experiments was so low that it was only just measurable, it can only be said that the hydrolysis rate of the residual dextrin S I is very low compared to that of starch. The ratio is certainly smaller than 1/10,000. A second series of experiments with a higher enzyme concentration was carried out.

Action of saliva preparations on limit dextrin fraction S II

The following mixtures were prepared and stored under toluene at 40°: 1.8 g dextrin (dry weight, i.e. ~ 2.0 g theoretical glucose), 1 ml phosphate buffer of pH = 6.9, 0.05 g NaCl, 25 ml saliva preparation. Increase of reducing power was determined as above (glucose as per cent of theoretical). Fermentation experiments were also carried out (Table 5). Paper chromatograms were prepared; a few are shown in Fig. 2.

It may be that the compositions of the reaction mixtures arrived at in these experiments do not represent equilibria or final states. In order to see if inactivation of the enzyme during the experiment could be responsible for the result, we determined the activity of the reaction mixtures after 290 days at 40° in new experiments with starch, comparable to those recorded in Table 2: 25 ml 2 per cent starch solu-

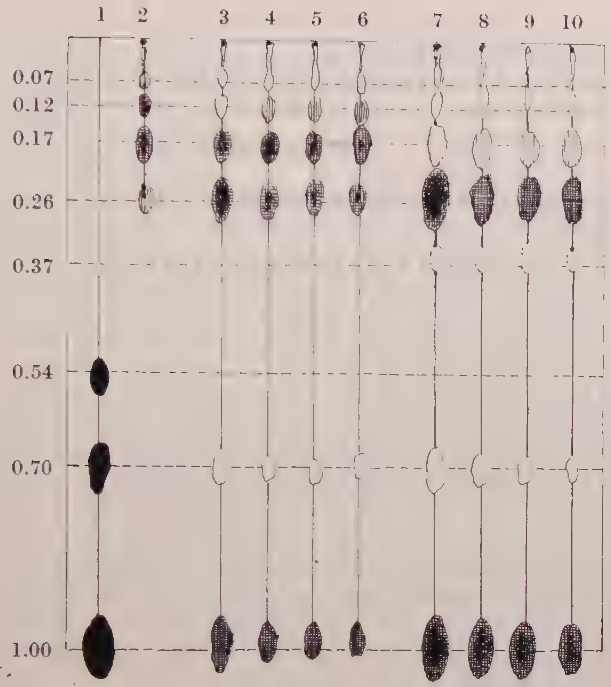


Fig. 2.

tion, 19 ml water, 5 ml phosphate buffer of pH = 6.9, 0.1 g NaCl and 1 ml of each reaction mixture. Increase of reducing power was determined as above and calculated as per cent glucose, Table 6.

A comparison between Tables 6 and 2 shows that, although the activity has decreased some 50 per cent, the amylase is still very active after 290 days at 40° and, furthermore, we find that the inactivation is about the same in all four experiments. This confirms earlier observations on the extraordinary stability of salivary amylase.

Table 5. Action of saliva preparations on fraction S II

Days	Reducing power; per cent glucose							
	Enzyme not heated		Enzyme heated to					
			63°		64°		65°	
	Red. power	Per cent glucose	Red. power	Per cent glucose	Red. power	Per cent glucose	Red. power	Per cent glucose
0	16.8	0	16.8	0	16.8	0	16.8	0
6	21.5	9	17.5	3	17.5	—	17.5	2
63	31.1	29	23.4	12	22.9	12	20.7	10
111	36.4	—	24.2	—	—	—	22.7	—
188	37.1	—	25.3	—	25.3	—	24.5	—
236	38.8	35	26.1	22	26.1	21	25.2	16

Table 6. Amylase activity of the reaction mixtures

Reaction time	Reducing power calculated as per cent gucose			
	Enzyme not heated	Enzyme heated to		
		63°	64°	65°
2 min.	3.3	—	1.6	—
5 „	7.9	4.0	3.2	3.0
10 „	13.4	7.0	6.6	5.1
20 „	20.7	12.9	12.9	10.8
24 hrs.	39.2	33.9	32.0	28.6

Cromatographical examination. According to the papergrams the substrate, limit dextrin fraction S II (No. 2 in Fig. 2), is composed of dextrins with 4, 5, 6, and 7 units together with unknown amounts of higher dextrins containing 8 or more glucose units (cf. also Fig. 1 in (13)). The hexasaccharide fraction appears to be the most abundant.

After *63 days' action* of the enzyme a set of papergrams was prepared (Nos. 3–6 in Fig. 2). Untreated solution of S II was put on as a reference (No. 2 in Fig. 2) and also a solution of glucose, maltose and isomaltose (No. 1 in Fig. 2). Inspection of the papergrams revealed the formation in all experiments of fairly large amounts of glucose, especially in the experiment with unheated enzyme (No. 3 in Fig. 2). Only small amounts of maltose could be observed; this may be due to the hydrolyzing action of the enzyme on maltose (Table 3). No formation of isomaltose or of a trisaccharide could be observed in any of the experiments, a further piece of evidence against the MEYER theory of isomaltose as the only true limit dextrin of all α-amylases.

In the experiments with unheated enzyme the tetrasaccharide fraction had increased considerably (No. 3 in Fig. 2). The spots corresponding to the higher dextrins, particularly those with 6 and 7 units have decreased and are only faintly visible on some chromatograms. In complete agreement with earlier results, these experiments show that the slow secondary hydrolysis of the residual dextrins is mainly a liberation of glucose, directly or partially via maltose, from limit dextrins with more than 5 units, whereby a tetrasaccharide is formed that is not attacked by the salivary amylase in reasonable time.

The three experiments with heated enzyme (Nos. 4–6 in Fig. 2) give much the same picture, only less marked. Glucose has been formed together with small amounts of maltose, but no isomaltose and no trisaccharides. The amount of tetrasaccharide has increased and that of the dextrins with 6 and 7 units has decreased. The difference between these experiments and that with unheated enzyme appears to be only quantitative.

After *236 days' action* of the enzyme new sets of papergrams were prepared, before and after removal of the fermentable sugars by baker's yeast (Nos. 7–10 in Fig. 2). Inspection of the papergrams corroborated the conclusions related above: Glucose has been formed in considerable amounts, but the amounts of maltose are small. The tetrasaccharide fraction has increased very strongly, especially in the experiments with unheated enzyme (No. 7 in Fig. 2), in which the spots corresponding to hexa- and heptasaccharides have practically vanished. No isomaltose has been formed and of trisaccharides at the most merely traces. The difference between the four experiments 7–10 is only quantitative and appears to be explainable on the basis of the varying amounts of active enzyme present in the various experiments.

Discussion

The experiments show that human saliva, and certain partially inactivated preparations thereof, have a weak action on salivary residual dextrins with 6 or more glucose units, possibly also on pentasaccharide dextrins. If the tetrasaccharide limit dextrin with $R_G = 0.264$ is attacked at all by the enzyme(s), which does not seem probable, it must be concluded that the rate of hydrolysis is extraordinarily low even compared to the rate of hydrolysis of residual dextrins with 6 or more units. Isomaltose could not be found in any experiment and only doubtful traces of trisaccharides. The enzyme preparations have also a weak action on maltose. If this hydrolysis is caused by a contaminating maltase it must be concluded that this hypothetical enzyme has approximately the same heat stability at pH 6.9 as has the salivary amylase. This must be valid also for the enzyme responsible for the hydrolysis of the residual dextrins with 6 or more glucose units. We are well aware that our experiments do not allow a definite answer to the question if the hydrolysis of the three substrates, starch, residual dextrins, and maltose, is caused by one, two, or three enzymes. We can only say that our experiments are compatible with the assumption that the hydrolysis of all three substrates is caused by the salivary amylase proper. If that is so, it seems natural to assume that maltotriose is also hydrolyzed at a low rate.

If the actions on different substrates observed by PAZUR (19) are thought to be caused by the salivary amylase it seems necessary to assume, as already mentioned, that the glucose formation takes place from the reducing chain-end. According to PAZUR the ratio of the hydrolysis rates of (*a*) the central linkages of maltotetraose, (*b*) the terminal linkage (or rather *one* terminal linkage) of this sugar, and (*c*) one linkage of maltotriose, is: 0.82:0.18:0.013. If the reducing end-group of maltotetraose is thus liberated about 14 times faster than the reducing end-group of maltotriose it seems reasonable to assume that, if the "reducing end-group" of maltose can also be liberated by the enzyme, the rate of this reaction should be still much lower. Even if glucose is not formed from maltose by *pure* salivary amylase, the enzyme can probably release this sugar from higher saccharides.

It may be pointed out that the stability against salivary amylase of some of the residual dextrins assumed to be stable by WHELAN and ROBERTS (16) appears to be questionable, not to say improbable. One is apt to ask, for instance, why in the case of the dextrins 6, 8, 10, and 12 in Fig. 1 one molecule of maltose is not split off from the "side chain". It will be remembered that even β-amylase is supposed to be capable of this reaction. The same may be said about liberation of maltose from the non-reducing end of the "main chain" of dextrins 7, 8, 11, and 12. Since dextrins of this type would be expected to occur in our residual dextrin preparations we have investigated the action of a very active soy bean β-amylase on residual dextrin fractions rich in higher dextrins. No measurable action was found, An amount of enzyme that hydrolyzes a 1 per cent starch solution to 16 per cent in 10 minutes, did not increase the reducing power of a similar dextrin solution in 3 days.

A few words might be said about the term "α-amylolysis" or "α-amylase action" used by many authors as if it were an axiom that all α-amylases have an identical action on their substrates. We have pointed out previously that this is not the case. At least in the earlier stages of starch hydrolysis the action of malt α-amylase differs markedly from that of other α-amylases, and it appears by no means certain that all α-amylases should have the same action on, e.g., the higher dextrins depicted in Fig. 1.

In this connection we should like to point out a mistake in a paper by WHELAN and ROBERTS (25) in which results on the hydrolysis of linear substrates by salivary amylase are reported. The authors find that their experiments are not in accordance with certain postulates in a paper by MYRBÄCK and SILLÉN (26). However, this paper deals with *malt* α-amylase and the calculations refer only to this enzyme. We have never assumed that they should be valid for salivary amylase, whose action, in our opinion, differs from that of malt α-amylase.

Summary

Residual dextrins produced from starch by salivary amylase have been isolated and roughly fractionated. Chromatographical examination showed the fractions to contain dextrins with 4–7 glucose units, some of them also still higher dextrins.

Certain residual dextrin fractions have been subjected to prolonged treatment with salivary amylase solutions, some of which had been partially inactivated by heating at 63–65°. In all experiments a very slow hydrolysis of residual dextrins with 6 or more units took place, probably also a hydrolysis of pentasaccharides. Glucose was liberated in this reaction and, also, to some extent, maltose.

No isomaltose is formed in this secondary hydrolysis, and of trisaccharides nothing or next to nothing. The tetrasaccharide residual dextrin fraction, on the other hand, increases strongly, and the amounts of higher residual dextrins, especially those with 6 and 7 glucose units, decrease, It is suggested that the tetrasaccharide is the lowest residual dextrin of salivary amylase and is really a *stable* dextrin.

It is suggested that *rapid* hydrolysis of starch (amylopectin) by salivary amylase yields the residual dextrins with 4–8 units, visualized in Fig. 1. Furthermore, it is suggested that in a *slow* reaction maltose is split off when possible from the non-reducing chain-ends.

Human saliva, or at least certain samples of human saliva, and partially heat-inactivated solutions of saliva as used in this investigation, have a weak but distinct action on maltose. It is not clear if this is an action of a contaminating maltase or of the amylase itself. Glucose is also formed from higher saccharides, presumably from the reducing end of the chain. This reaction, which is probably characteristic of the pure amylase, is also involved in the slow secondary saccharification of the "primary" residual dextrins.

Institute of Organic Chemistry and Biochemistry, University, Stockholm.

REFERENCES

1. MYRBÄCK, K., Advances Carbohydrate Chem. *3*, 252 (1942).
2. MYRBÄCK, K. and AHLBORG, K., Svensk Kem. Tidskr. *49*, 216 (1937); MYRBÄCK, K., Current Sci. India *6*, 47 (1937).
3. MYRBÄCK, K. and AHLBORG, K., Biochem. Z. *307*, 69 (1940).
4. AHLBORG, K. and MYRBÄCK, K., Ibid. *308*, 187 (1941).
5. MYRBÄCK, K., ÖRTENBLAD, B., and AHLBORG, K., Ibid. *307*, 53 (1941).
6. PRINGSHEIM, H. *et al.*, Biochem. Z. *142*, 108 (1923); *148*, 336 (1924); *173*, 399 (1926); *177*, 406 (1926); *197*, 143 (1928); *203*, 88 (1928).

7. Pan, S. C., Nicholson, L. W., and Kolachow, P., Arch. Biochem. Biophys. *42*, 421 (1953).
8. Bernfeld, P. and Fuld, M., Helv Chim. Acta *31*, 1423 (1948); Meyer, K. H., Duckert, F., and Fischer, E. H., Ibid. 33, 207 (1950); Meyer, K. H. and Gonon, F. W., Ibid. *34*, 294, 308 (1951).
9. Montgomery, E. M., Weakley, F. B., and Hilbert, G. E., J. Am. Chem. Soc. *69*, 2249 (1947).
10. Weinmann, H., J. Biol. Chem. *164*, 7 (1946); cf. also Tsuchiya, H. M., Montgomery, E. M., and Corman, J., J. Am. Chem. Soc. *71*, 3265 (1949); Phillips, L. L. and Caldwell, M. L., Ibid. *73*, 3363, 3559 (1951).
11. Myrbäck, K. and Svanborg, K., Arkiv Kemi *5*, 317 (1953).
12. Myrbäck, K. and Willstaedt, E., Arkiv Kemi *6*, 443 (1953).
13. Myrbäck, K., Arkiv Kemi *7*, 53 (1954).
14. Myrbäck, K. and Willstaedt, E., Arkiv Kemi *6*, 417 (1953).
15. Roberts, P. J. P. and Whelan, W. J., Biochem. J. (London) *49*, lvi (1951); *51*, xviii (1952).
16. Whelan, W. J. and Roberts, P. J. P., Nature *170*, 748 (1952).
17. Myrbäck, K. and Leissner, E., Arkiv Kemi, Mineral. Geol. *A 18*, No. 18 (1943).
18. Hopkins, R. H. and Bird, R., Biochem. J. (London) *54*, xxxvi (1953).
19. Pazur, J. H., J. Biol. Chem. *205*, 75 (1953).
20. Myrbäck, K. and Nycander, G., Biochem. Z. *311*, 234 (1942).
21. Svanborg, K. and Myrbäck, K., Arkiv Kemi *6*, 113 (1953).
22. Myrbäck, K. and Willstaedt, E., Arkiv Kemi *6*, 465 (1953).
23. Myrbäck, K. and Willstaedt, E., Arkiv Kemi *7*, No. 34 (1954).
24. Myrbäck, K., Biochem. Z. *307*, 140 (1941).
25. Whelan, W. J. and Roberts, P. J. P., J. Chem. Soc. *1953*, 1298.
26. Myrbäck, K. and Sillén, L. G., Svensk Kem. Tidskr. *56*, 60 (1944).

Tryckt den 20 augusti 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 46

Mitgeteilt am 10. Februar 1954 durch THE SWEDBERG und ARNE FREDGA

Elektronenmikroskopische und röntgenographische Studien an Superpolyamidfäden. Beziehungen zwischen dem Feinbau der Superpolyamidfäden und dem der Viscoseseide

Von EDGAR RIBI[1] und ANNALISA NORLING

Mit 4 Figuren im Text

Beim Vergleich der heutigen Vorstellungen über die submikroskopische Struktur der Superpolyamidfäden mit derjenigen der Cellulosefasern, im besondern der regenerierten Cellulose, der Viscoseseide, zeigen sich auffallende Analogien. Die Kenntnisse über den Feinbau der Superpolyamidfäden sind vor allem eingehenden Röntgenanalysen zu verdanken. Im Falle der Viscoseseide haben ausser den röntgenographischen Strukturuntersuchungen auch elektronenmikroskopische Studien erlaubt, den Einblick in deren Feinbau zu vertiefen. Es schien daher von Interesse, ebenfalls an den Superpolyamidfäden Beobachtungen mit Hilfe des Elektronenmikroskopes anzustellen. Dabei waren Ergebnisse zu erwarten, welche nicht nur wertvolle Vergleiche mit den feinbaulichen Verhältnissen der Viscoseseide gestatten sollten, sondern auch ganz allgemein erweiterte Vorstellungen über die Natur der Faserstoffe.

Die Ermittlung der röntgenograpischen Feinstruktur ergab, dass stark verstreckte Superpolyamidfäden zu einem Faserdiagramm führen. Die linearen Makromoleküle (paraffinartige Zickzackketten) verlaufen parallel der Faserachse und liegen zu einem Makromolekülgitter vereinigt vor; ähnlich der Cellulose ist den Superpolyamidfäden ein mizellarer Aufbau eigen (1, 2, 3, 4).

Schon eine erstarrte Superpolyamidschmelze weist einen mikrokristallinen Charakter auf. Nach HESS und KIESSIG (4) sind die Interferenzen der Röntgendiagramme von erstarrten Schmelzen nicht weniger scharf als diejenigen von gedehnten Fäden. Analoge Verhältnisse sind beim Vergleich der Röntgenaufnahmen der isotropen Viscosefäden (HERMANSsche Modellfäden) mit denjenigen der verstreckten Viscosefäden gefunden worden (5, 6, 7). Die kristallinen Bereiche in den erstarrten Superpolyamidschmelzen besitzen nach Schätzungen von v. ARDENNE, SCHIEBOLD und GÜNTHER (8) Ausdehnungen von der Grössenordnung 100 Å. Dieselbe Grössenordnung ergab sich für die Länge der kristallinen Bereiche der isotropen Viscosefäden beim qualitativen Vergleich der Schärfe der Röntgen- mit denjenigen der Elektroneninterferenzen (7). Die erwähnten Analogien lassen daher für die erstarrten Superpolyamidschmelzen ähnliche submikroskopisch morphologische Verhältnisse erwarten, wie sie bei den isotropen Viscosefäden elektronenmikroskopisch beobachtet wurden (7).

---

[1] Adresse: National Institute of Health, Rocky Mountain Laboratory, Hamilton, Montana, U. S. A.

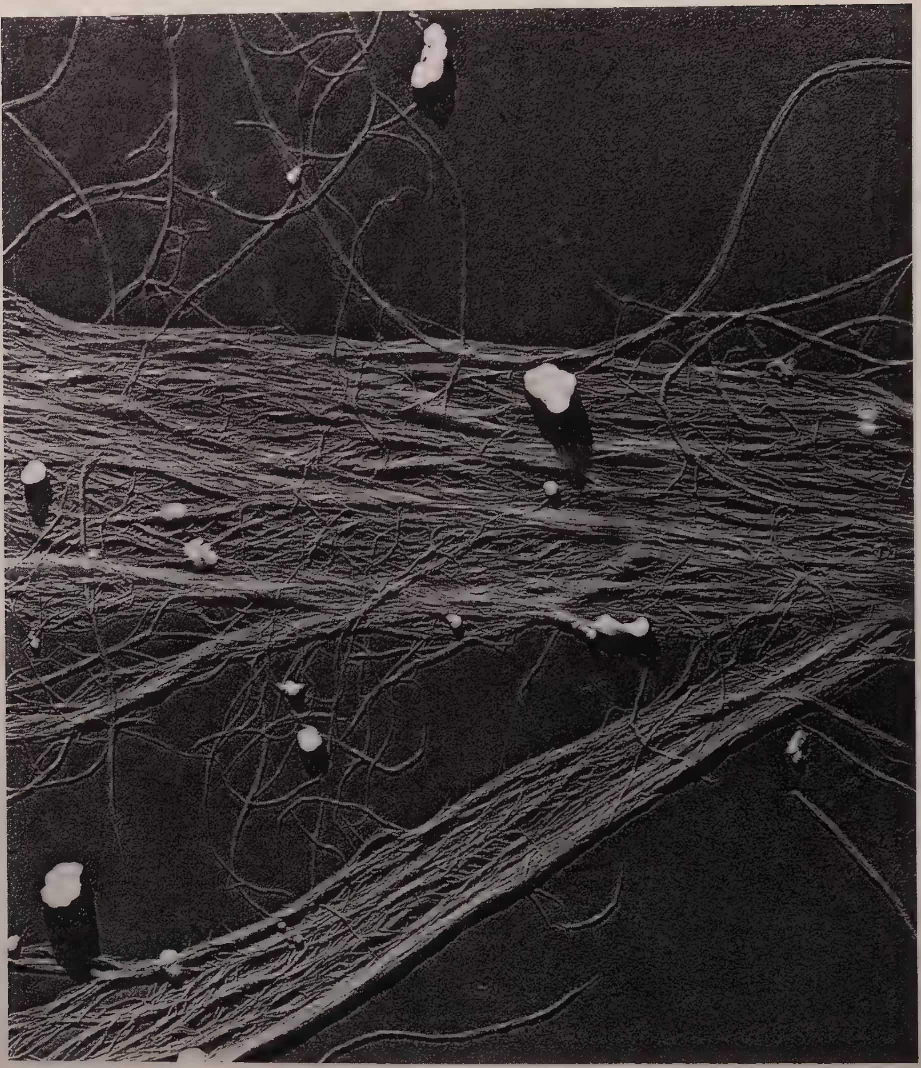


Fig. 1. Faden eines Superpolyamides aus ε-Aminocapronsäure (Probe B) mit Ultraschall zerkleinert. 21 000 ×.

Hess und Kiessig (4) fanden an den Röntgendiagrammen der verstreckten Superpolyamidfäden Reflexe, welche auf grosse Perioden im Bereiche von 100 Å hinweisen. Diese Interferenzen sind offenbar unabhängig von dem normalen Kristallgitter der Fäden und werden mit sehr gleichmässigen Längen der gittermässig geordnete Bereiche in Zusammenhang gebracht. Aus dem qualitativen Vergleich der Röntgen- mit den Elektroneninterferenzdiagrammen, sowie auf Grund der Mikroskopierung der beim selektiven hydrolytischen Abbau von Fasern angereicherten Kristallite, wurde ebenfalls für die Länge der kristallinen Bereiche ver-

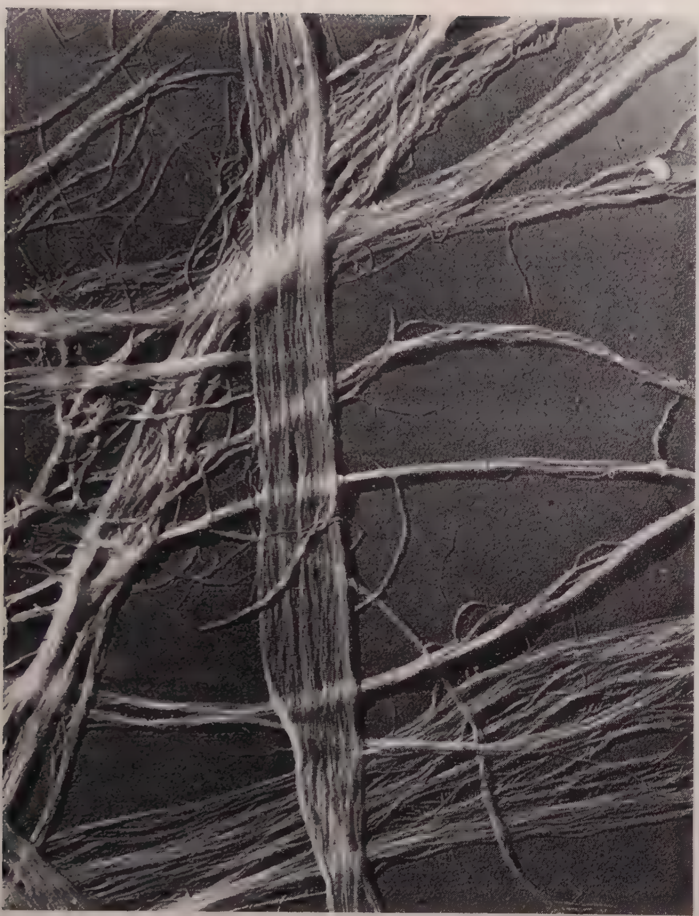


Fig. 2 Handelsüblicher Nylonfaden mit Ultraschall zerkleinert. 21 000 ×.

streckter Viscosefäden eine Grösse im Bereiche von 100 Å angenommen (7).[1] Reflexe von langen Perioden, wie sie an den Röntgendiagrammen von verstreckten Superpolyamidfäden auftreten, liessen sich bei der Viscoseseide bisher nicht beobachten. Es ist anzunehmen, dass die Schwankungen der Längenausdehnungen der Mizellen bei der regenerierten Cellulose zu gross sind, um das Entstehen von Langperiodeninterferenzen zu veranlassen.

Aus der charakteristischen Verschmierung der Langperiodenreflexe, welche nicht auf eine unvollständige Faserorientierung der Superpolyamide zurückzuführen ist, schliessen HESS und KIESSIG (4) auf die Anwesenheit dünner Kristallfäden mit Dicken, die unter hundert Å liegen und möglicherweise lediglich aus Bündeln von einigen wenigen geordnet zusammengelagerten Molekülfäden bestehen. Über-

---

[1] Die auf osmotischem Wege ermittelten Durchschnittskettenlängen bei den technischen Superpolyamidfäden betragen wie bei den Viscoseseiden grössenordnungsmässig tausend Å (9, 10). Für beide Fasersysteme ist daher zu erwarten, dass ein Fadenmolekül gleichzeitig mehrere Mizellen durchziehen kann.

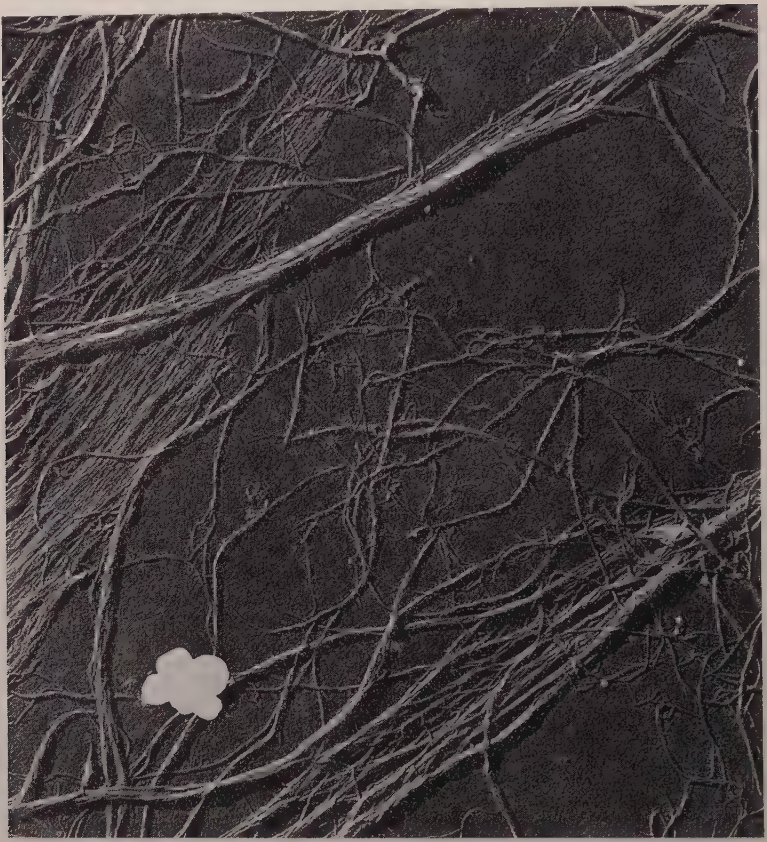


Fig. 3. Plastik aus Polyäthylen mit Ultraschall zerkleinert. 25 500 ×.

einstimmend bringen dieselben Verfasser auch die beobachtete äquatoriale Kleinwinkelstreuung in Zusammenhang mit der Existenz einer feinfibrillären Struktur. Eine quantitative Auswertung der Kleinwinkelstreuung von Fankuchen und Mark (11) führte zur Annahme einer zwischen 200 und 300 Å liegenden Periodizität oder Quasiperiodizität senkrecht zur Achse der verstreckten Superpolyamidfäden. Dieselbe Methode ergab für eine hochorientierte Viscoseseide zwischen 50 und 200 Å schwankende Breiten der kristallinen Breiche (11). In der Folge zeigte eine elektronenmikroskopische Untersuchung, dass dem Aufbau der Viscoseseide ein Elementarfibrillensystem zugrundeliegt. Auf den Mikroskopaufnahmen konnte diskrete Fibrillen mit recht einheitlicher Dicke im Bereiche von hundert Å festgestellt werden (7).

Sämtliche erwähnten Analogien liessen vermuten, dass mit Hilfe der Elektronenmikroskopes bei den Superpolyamidfäden das Vorliegen eines der Viscoseseide ganz analogen Systems von submikroskopischen Fibrillen auf direktem Wege nachgewiesen werden kann. Figur 1 zeigt ein bandartiges Fragment eines, mit Hilfe des Ultraschalls zerkleinerten, kaltverstreckten Superpolyamidfadens aus einem Kondensat von ε-Aminocapronsäure (im folgenden als Probe B bezeichnet).

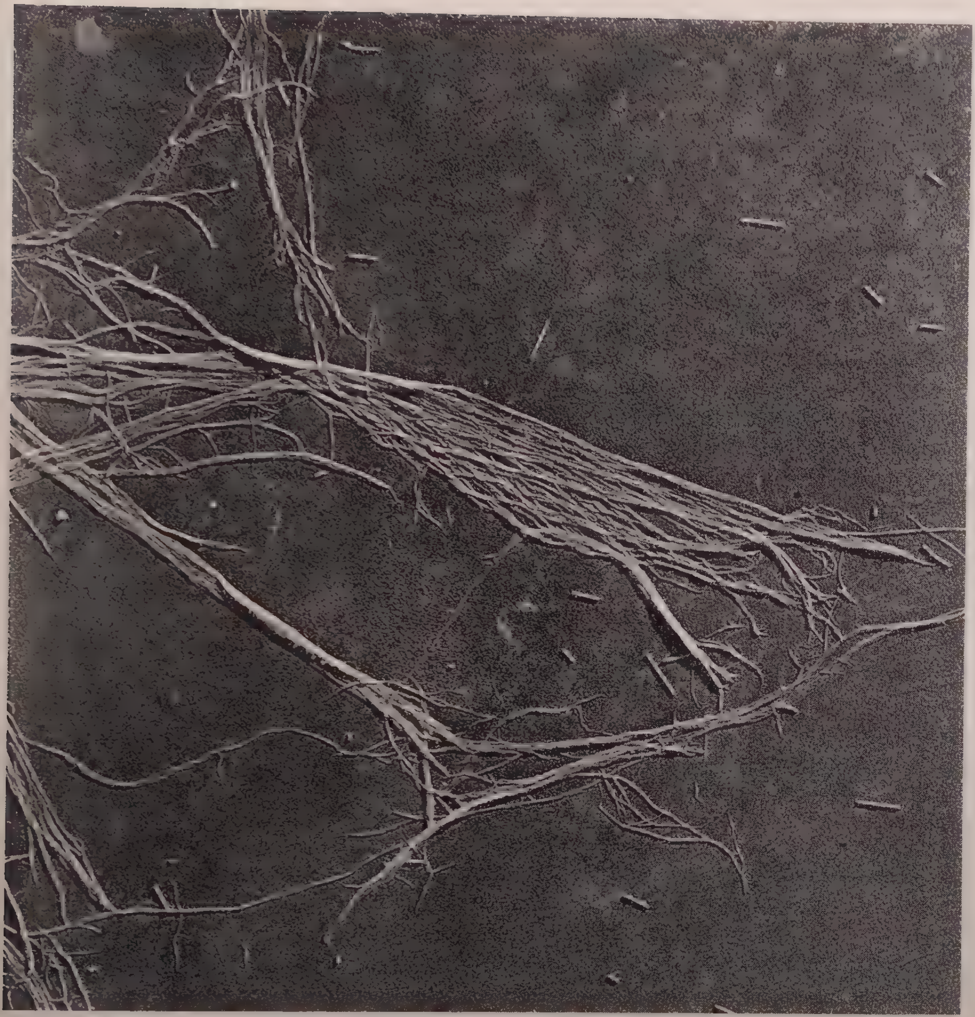


Fig. 4. Holzcellulose mit Ultraschall zerkleinert und Teilchen von Tabakmosaikvirus. 21 000 ×.

Superpolyamidfäden lassen sich zur elektronenmikroskopischen Beobachtung geeignet zerkleinern, indem die durch Schaben der Fäden mit Hilfe einer scharfen Glaskante erhaltenen und in Wasser aufgeschlämmten feinsten Späne einem intensiven Ultraschallfeld ausgesetzt werden.

Das abgebildete Fragment stellt ein Aggregat von diskreten submikroskopischen Fibrillen dar, deren Dicke im Bereiche von 100 Å liegt. Erwartungsgemäss liessen sich auch die Defibrillierungsprodukte von handelsüblichen Nylonfäden verschiedener Herkunft stets als Bündel oder bandartige Aggregate von Elementarfibrillen identifizierten (Fig. 2). Die Abbildungen sind sehr ähnlich denjenigen der bandartigen Sekundärwandfragmente, die bei der mechanischen Zertrümmerung von pflanzlichen Zellwänden mit Hilfe des ,,Turmix" erhalten werden (Fig. 4) (12, 13, 14). Ebenfalls als durchgehend aus Elementarfibrillen aufgebaut erwies sich ein aus der Schmelze von polymerisierter ε-Aminocapronsäure erzeugter und in der Wärme gezogener Faden (im folgenden als Probe A bezeichnet).[1] Während Probe B zu einem guten Faserdiagramm führte, wiesen die Röntgeninterferenzen der Probe A darauf hin, dass hier die kristallinen Bereiche offenbar nur teilwise in der bevorzugten Lagerung der Fadenachse vorhanden waren. Die übrigen kristallinen Bereiche sind entweder ungeregelt oder bilden eine pseudostatistische Textur.

Die Röntgenaufnahme der Probe A unterscheidet sich etwas von den Diagrammen, welche Brill (15) sowie Müller und Herbst (16) für den Übergang zwischen regellosen und geregelten Kristalliten beschrieben. Von den beiden intensiven Reflexen (den Netzebenenabständen von 3,6 Å und 4,3 Å entsprechend) erscheint auf der Aufnahme der Probe A die innere Linie nicht peripher homogen geschwärzt und die äussere Interferenz ist nicht vollkommen in Segmente aufgespalten. Wohl tritt die dem kleineren Beugungswinkel entsprechende Linie längs ihres ganzen Umfanges mit einer beträchtlichen Intensität auf, zeigt aber am Äquator eine Sichel als zusätzliche Verstärkung überlagert. Die unter dem grössern Beugungswinkel auftretende Interferenz besteht aus sehr ausgeprägten Sicheln. Zwischen den letzteren ist aber die Inteferenzlinie nicht verschwunden und erscheint mit einer gewissen Intensität. Danach ist die Segmentaufspaltung im Falle der Probe A keine partielle. Es sind einerseits die dem äussern Reflex entsprechenden Netzebenen noch nicht vollkommen der Fadenachse parallel gestellt, anderseits sind die der innern Linie entsprechenden Netzebenen in ihrer Mannigfaltigkeit der Lagen bereits beschränkt.

Unterschiede in den Elektronenmikroskopaufnahmen zwischen den Proben A und B bestanden lediglich im ausgeprägteren Auftreten von bandartigen Spaltstücken beim kaltverstreckten Superpolyamid. Charakteristische Differenzen zwischen den Fibrillendicken der beiden Proben konnten nach Ausmessen an den Originalplatten (Mikroskopvergrösserung 8 500 ± 2,5%) mit Hilfe eines Mikrokomparators und statistischer Auswertung der Messresultate nicht festgestellt werden. Hess und Kiessig (4) schlossen bekanntlich aus ihren Röntgenaufnahmen, dass die Dicke der kristallinen Bereiche der Superpolyamide mit zunehmender Verstreckung der Fäden abnimmt. Die Genauigkeit der angewendeten elektronenmikroskopischen Methode genügt offenbar nicht, um wirklich bestehende Dickendifferenzen zu erfassen.

Im Zusammenhang mit der Beobachtung, wonach die Probe A bereits von Elementarfibrillen durchzogen wird, sei auf ähnliche Resultate anlässlich elektronenmikroskopischer Studien an Viscoseseiden hingewiesen (7).

Viscosefäden, die beim Verspinnen lediglich eine Verstreckung von 1% erlitten (Probe V_{IV}), wurden als durchgehend aus Elementarfibrillen aufgebaut beschrieben (7). Nachträglich hergestellte Röntgenaufnahmen führten zu Diagrammen mit Kreisinterferenzen ohne Anzeichen von verstärkten Segmenten.[2] Elektronenmikroskopisch unterschieden sich die Elementarfibrillen der Probe V_{IV} nicht von den Fibrillen jener Fäden, welche unter 70%iger Verstreckung gesponnen wurden (Probe V_V). Das Röntgendiagramm der Probe V_V zeigte sehr kurze Segmente, zwischen welchen die Interferenzlinien völlig verschwanden.

---

[1] Die Superpolymeren aus ε-Aminocapronsäure (Proben A und B) wurden von der Svenska Oljeslageri AB, Göteborg, Schweden, zur Verfügung gestellt, wofür wir unseren Dank aussprechen.

[2] Anlässlich der früheren Untersuchungen an Viscoseseiden (7) wurden lediglich Röntgenaufnahmen der absichtlich desorientierten Viscosefäden hergestellt, um durch Diagrammvergleich Unterschiede im Kristallinitätsgrad zwischen verschiedenen Seideproben und deren pulverförmigen Hydrolyseprodukten festhalten zu können.

Obwohl die Röntgenaufnahme der Probe V_V auf einen isotropen Zustand hinweist, sind mehr oder weniger gut parallel der Drahtachse gelagerte kristalline Bereiche nicht ausgeschlossen. Zu berücksichtigen ist, dass zur Herstellung des Diagrammes ein makroskopischer Teil des Seidefadens durchstrahlt wurde. Es dürfte mit den von Kratky (17) an Hydratcellulosefilmen und den von Hermans (18) an Hydratcellulosefäden diskutierten „Ordnungen in kleinsten Bereichen" gerechnet werden: Bei der Annahme einer „verwackelten Parallelität" entsteht die Mannigfaltigkeit der Lagen in einem makroskopisch oder lichtmikroskopisch sichtbaren Gebiete dadurch, dass sich beim Fortschreiten im Raume von einem kristallinen Bereiche zum andern die gegenseitigen Verwerfungen summieren. Eine auf diese Weise entstandene Lagenmannigfaltigkeit kann sich im Röntgendiagramm analog äussern, wie ein Präparat mit völlig ungeregelten kristallinen Bereichen.

Man gelangt zur Vorstellung, dass beim Verfestigen geschmolzener Superpolyamide oder gelöster Cellulose relativ kleine Orientierungseffekte genügen, um die Bildung von Elementarfibrillen zu verursachen.

Ein Vergleich der Debye-Scherrer-Diagramme der pulverisierten Superpolyamidproben A und B untereinander zeigte keine Differenzen in den Intensitäten sämtlicher Linien oder der durch die diffuse Streustrahlung hervorgerufenen Allgemeinschwärzung des Filmes.

Pulverisierte Proben konnte durch Schaben der Polyamidfäden mit scharfen Glaskanten erhalten werden. Die Pulver wurden zu Stäbchen mit gleichem Durchmesser und gleicher Schüttdicke gepresst, in derselben Kamera unter übereinstimmenden Bedingungen geröntgt und schliesslich die Filme gemeinsam entwickelt. Das Einhalten der gleichen Versuchsbedingungen erlaubte, die Diagramme der Proben A und B in bezug auf die Intensität ihrer Interferenzen und der Untergrundschwärzung miteinander zu vergleichen.

Infolge der nicht sehr grossen Genauigkeit der angewandten röntgenographischen Methode kann trotz des beobachteten analogen Interferenzvermögens ein der Nachweisbarkeitsgrenze ensprechender Unterschied im Kristallinitätsgrad nicht ausgeschlossen werden.

Fankuchen und Mark (11) schliessen aus den mit Hilfe einer Mikromethode hergestellten Röntgendiagrammen, dass im Innern eines unverstreckten Drahtes das Superpolyamid unorientiert und im mesomorphen Zustand vorliegt, während eine Oberflächenschicht aus unorientierten Kristalliten besteht. Bei der Verstrekkung erfolgt im Innern des Fadens der Übergang von mesomorpher in kristalline Phase und Orienterung; an der Oberfläche werden die bereits vorhandenen kristallinen Bezirke orientiert. Hess und Kiessig (4) nehmen auf Grund ihrer röntgenographischen Studien an, dass beim kalten Verstrecken der Superpolyamide parallel dem Orientierungsvorgang eine völlige Umordnung der gittermässig geordneten Bereiche stattfindet, d. h. ein Abbau und Neuaufbau derselben. Eine Zunahme der Kristallisation bei der Verstreckung stellten die letztgenannten Autoren nicht fest, hingegen, wie bereits erwähnt, eine veringerte Dicke und Länge an den umgebildeten kristallinen Bereichen.

Die mikroskopischen Beobachtungen an unseren Proben führen zur Vorstellung, dass beim Übergang von A in B die Erhöhung des Orientierungsgrades einer Orientierung von diskreten Elementarfibrillen gleichkommt, in welchen die von

Fankuchen und Mark sowei Hess und Kiessig mitgeteilten Veränderungen erfolgen können.

Im Zusammenhang mit den bei der regenerierten Cellulose gefundenen feinbaulichen Verhältnissen schien es von besonderem Interesse, auch die aus Superpolyamidlösungen erzeugten Fäden elektronenmikroskopisch zu untersuchen.

Zu diesem Zwecke wurden m-Kresol und Phenollösungen von laboratoriumsmässig hergestellter Polyaminocapronsäure und handelsüblicher Nylonfasern in Wasser gedüst und die Fällungsprodukte zu Fäden ausgezogen.

Nach der mechanischen Defibrillierung (Ultraschallbehandlung) der auf diese Weise erhaltenen Fäden konnten unter dem Elektronenmikroskop die üblichen Elementarfibrillen beobachtet werden: einzelliegend, zu Bündeln und Bändern vereinigt. Dieses Ergebnis kann auch bei Fäden erhalten werden, deren Kresolausgangslösungen Konzentrationen aufweisen, die für osmotische oder viskosimetrische Ermittlungen der Molekulargewichte angewendet werden.

Fäden aus einer 0,5–1%igen Lösung von Superpolyamid in m-Kresol lassen sich wie folgt herstelllen: Die Lösung wird aus einer Glaskapillare sorgfältig auf die Oberfläche von warmem Wasser gedüst. Die sich auf der Wasseroberfläche bildende Fällung, welche die Form eines Folienbandes aufweist, lässt sich zu Fäden ausziehen.

Nach der Schule Staudingers hat man anzunehmen, dass in einer 0,5–1%-igen m-Kresollösung das Superpolyamid molekulardispers vorliegen sollte. Danach wäre die Bildung des submikroskopischen Fibrillensystems aus der sehr verdünnten Kresollösung nicht auf eine unvollständige Auflösung der Ausgangsfaser zurückzuführen.

Die bei den Superpolyamiden gemachten Erfahrungen erlauben einige Betrachtungen zum Problem über die Bildung der Viscoseseide. Auf Grund der gefundenen submikroskopischen Ausbildungsformen der Viscoseseide und deren Konstanz in ihrer Kristallinität (6, 7) sollte man vorerst geneigt sein, die feinbaulichen Verhältnisse der wiedergefällten Cellulose in Zusammenhang mit Resten kristalliner Bereiche von nativer Cellulose zu bringen, welche über den Viscoseprozess herübergerettet werden. Dass aber zur Entstehung eines Mizellargerüstes, wie es der Cellulose eigen ist, nicht kristalline Bereiche der ursprünglichen Faser vorausgesetzt zu sein brauchen, zeigt die Bildung der Superpolyamidfäden, die aus Schmelzen von vollsynthetisch hergestellten Fadenmolekülen gewonnen wurden. Da auch das laboriatoriumsmässige Verarbeiten von 0,5–1-%igen Lösungen von Superpolyamidfäden in m-Kresol wiederum zu Fäden mit einem dem Ausgangsmaterial analogem Feinbau führt, dürfte man annehmen, dass ebenfalls im Falle der regenerierten Cellulose die Eigenschaften der Fadenmoleküle *selbst* genügen könnten, um beim Fällen von gelöster Cellulose die Entstehung einer Mizellarstruktur zu veranlassen. Zu dieser Annahme führten bereits die Versuche von Kratky und Treiber (19), unter der Voraussetzung der Gültigkeit der Auffassung der Schule Staudingers, wonach in sehr verdünnter Viscose die Cellulose molekular gelöst vorliegt. Kratky und Treiber verdünnten technische Viscose auf das ungefähr 20fache. Nach Wiederaufkonzentrieren im Vakuum wurden Hermanssche Modellfäden hergestellt. Letztere wiesen in erster Näherung dieselbe Kristallinität auf wie eine direkt aus der ursprünglichen Viscose erzeugte Modellfaser.

Beschränkten Umfanges sind elektronenmikroskopische Studien auch auf das vom geometrischen Standpunkt aus gesehen einfachste linearpolymere System, das vollsynthetische Polyäthylen, ausgedehnt worden. Figur 3 zeigt die unter dem Elektronenmikroskop beobachteten Ausbildungsformen von feinsten Zertrümmerungsprodukten eines aus Polyäthylen erzeugten dünnen Katheterschlauches.[1]

In Wasser aufgeschlämmte feinste Späne, die durch Schaben des Polyäthylenschlauches mit Hilfe scharfer Glaskanten erhalten wurden, liessen sich teilweise mit intensivem Ultraschall zur elektronenmikroskopischen Untersuchung geeignet fein zerkleinern.

Auch hier hat man sich vorzustellen, dass sich lineare Makromoleküle bei ihrer Kristallisation bevorzugt zu diskreten submikroskopisch Fibrillen vereinigen. Letzteres offenbar stets dann, wenn die Kristallisation der Fadenmoleküle von äusseren Orientierungseffekten begleitet wird, was auch bei Herstellung des untersuchten Polyäthylenplastiks der Fall war. Objektive Dickenunterschiede zwischen den Fibrillen der Superpolyamide und denjenigen des Polyäthylens konnten beim Ausmessen an den Mikroaufnahmen mit Hilfe eines Mikrokomparators wiederum nicht festgestellt werden. Die Messresultate erlauben lediglich auszusagen, dass die Dicke der Fibrillen dieser beiden vollsynthetischen Produkte im Bereiche von rund hundert Å liegt. Dasselbe Ergebnis wurde nicht nur bei der elektronenmikroskopischen Ermittlung der Dicke von feinsten Viscoseseidefibrillen, sondern auch bei den Fibrillen der nativen Holzcellulose, der nativen und mercerisierten Baumwolle und des Chitins gefunden. Tatsächlich extierende, bestimmte Differenzen zwischen den Elementarfibrillendicken der erwähnten Faserstoffe müssten, wie bereits erwähnt, ausserhalb der Erfassungsgrenze der angewandten elektronenmikroskopischen Methode liegen. Letzteres dürfte auch die Ursache dafür sein, dass sich eine bändchenförmige Gestalt der isolierten Elementarfibrillen nicht festhalten liess, wie auf Grund der postulierten Blättchennatur der Cellulose- und Superpolyamidmizellen zu erwarten gewesen wäre (15, 20). Nach KRATKY und Mitarbeitern (21) könnte das Futteral eines Rechenschiebers etwa die Dimensionsverhältnisse der Mizellen veranschaulichen.

Um zu demonstrieren, dass die Dicken der feinsten Fibrillen der von uns bisher untersuchten Faserstoffe im Bereiche von ungefähr 100 Å liegen, wurde Figur 4 beigefügt. Es handelt sich um die Abbildung eines Fragmentes der Sekundärwand einer Holzfaser und um Teilchen des Tabakmosaikvirus. Die Dicke der Virusstäbchen liegt nach eigenen Ausmessungen zwischen 150 und 200 Å. Aus der Abbildung geht zweifellos hervor, dass die Cellulosefibrillen dünner sind als die ,,Mikrofibrillen'', welche FREY-WYSSLING (22) beschreibt. Auch an Mikrotomschnitten von Holzgewebe konnte elektronenmikroskopisch dieselbe Fibrillendicke identifiziert werden wie die Dicke der Fibrillen von Holzfasern nach deren mechanischer Zertrümmerung mit Hilfe des Ultraschalls (23). Nach Abschluss der hier beschriebenen Untersuchungen wurde eine Präparationsmethode angewandt, die den Elementarfibrillenbau von Fasersystemen elektronenmikroskopisch festzuhalten erlaubt, ohne auf eine mechanische oder chemische Zerkleinerung der Faserstoffe angewiesen zu sein. Hierüber soll in einer folgenden Mitteilung berichtet werden.

Professor THE SVEDBERG und Professor STIG CLAESSON danken wir für ihr grosses Interesse, welches das Zustandekommen dieser Arbeit ermöglichte. Herrn GÖTE EKLUND sind wir für

---

[1] Hergestellt von CLAY-ADAMS COMPANY, New York, U.S.A.

wertvolle technische Assistenz dankbar. Den schwedischen Cullulosefirmen *Billeruds AB, Mo och Domsjö AB, Stora Kopparbergs Bergslag AB, Svenska Cellulosa AB* und *Uddeholms AB* danken wir für ihre finanzielle Unterstützung.

Uppsala, Physikalisch-chemisches Institut.

LITERATURVERZEICHNIS

1. Carothers, W. H., und Hill, J. W., J. Am. Chem. Soc. *54*, 1579 (1932).
2. Fuller, C. S., Chem. Rev. *26*, 143 (1940); Fuller, C. S., Baker, W. O., und Pape, N. R., J. Am. Chem. Soc. *62*, 3275 (1940).
3. Brill, R., Naturwissenschaften *29*, 220 (1941); J. prakt. Chem. *161*, 49 (1942); Z. physik. Chem. B *53*, 61 (1943).
4. Hess, K., und Kiessig, H., Z. physik. Chem. *193*, 196 (1944).
5. Kratky, O., und Sekora, A., Kolloid-Z. *108*, 169 (1944).
6. Hermans, P. H., und Weidinger, A., J. Polymer Sci. *4*, 135 (1949).
7. Ribi, E., Arkiv Kemi *2*, 551 (1950).
8. v. Ardenne, M., Schiebold, E., und Günther, F., Z. Physik *119*, 352 (1942).
9. Staudinger, H., und Jörder, H., J. prakt. Chem. *160*, 176 (1942).
10. Enoksson, B., unveröffentlichte Resultate; mitgeteilt von E. Ribi, E., Arkiv Kemi *2*, 551 (1950).
11. Fankuchen, I., und Mark, H., J. Applied Phys. *15*, 364 (1944).
12. Mühlethaler, K., Biochim. et Biophys. Acta *3*, 15 (1949).
13. Rånby, B. G., und Ribi E., Experientia *6*, 12 (1950).
14. Hodge, A. J., und Wardrop, A. B., Australian J. Sci. Res. B *3*, 265 (1950).
15. Brill, R., Z. physik. Chem. B *53*, 61 (1943).
16. Müller, A., und Herbst, M., Kunststoffe *41*, 145 (1951).
17. Kratky, O., Kolloid-Z. *68*, 347 (1934).
18. Hermans, P. H., Kolloid-Z. *83*, 71 (1938).
19. Kratky, O., und Treiber, E., Diskussionsbemerkung von O. Kratky zur Arbeit von P. H. Hermans und A. Weidinger, Kolloid-Z. *115*, 103 (1949).
20. Kratky, O., und Mark, H., Z. physik. Chem. B. *36*, 129 (1937).
21. Kratky, O., Sekora, A., und Treer, R., Z. Elektrochem. *48*, 587 (1942); Kratky, O., Porod, G. und Treiber, E., Z. Elekrochem. *55*, 481 (1951).
22. Frey-Wyssling, A., Makromol. Chem. *6*, 7 (1951).
23. Ribi, E., Exp. Cell Res. *5*, 161, 1953.

Tryckt den 20 augusti 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 47

Communicated 14 April 1954 by ARNE WESTGREN and ARNE TISELIUS

Solubility differences of desoxyribonucleoprotein prepared from different sources

By GÖSTA FRICK

With 5 figures in the text

Introduction

In one of their first papers on the now well-known method of preparing desoxyribonucleoprotein (DNP) MIRSKY and POLLISTER (1) point out that the DNP from different animals may differ in their solubility in NaCl solutions.

They found that instead of the usual 1 M NaCl it was necessary to use 2 M NaCl to bring the DNP of sea urchin sperm into solution. The DNP from dogfish, it is also stated, precipitates at a higher NaCl concentration than the DNP from mammalian tissue. MIRSKY and POLLISTER drew the conclusion that "The solubility of the nucleoprotein is probably correlated with the salt concentration of the body fluids".

The aim of the work presented in this article is a more detailed investigation of the solubility of DNP prepared from animals with different "milieu intérieur". The tissue used was liver from calf, marine bony fish and marine elasmobranch.

It was found desirable first to investigate the effect of employing different ionic strengths during the preparation on the solubility behaviour of the DNP from a given species.

There will also be a short discussion of some observations on the solubility behaviour of the DNP, which probably are due to the enzymatic activity of the tissue.

Preparation of desoxyribonucleoprotein (DNP)

The DNP was extracted according to MIRSKY and POLLISTER and purified by repeatedly dissolving the material in 1 M NaCl and subsequently precipitating it by dilution to 0.14 M. The preparation was carried out in the cold. For other details see earlier articles (2, 3).

One difference from this earlier described method is that the final solution was buffered with only 0.01 ionic strength phosphate to pH 6.3, whereas the first redissolvings of DNP in 1–2 M NaCl were buffered with phosphate of 0.05 ionic strength.

The usual way to collect the precipitate is to wind it along a rod and just lift it up from the mother liquid. In the present study the precipitate has in some cases been collected by centrifugation instead. This will be pointed out for each such case.

In order to save material the number of precipitations of the DNP preparations from fish liver was only three or four instead of the usual six or eight.

The precipitation curves

The precipitation curve—amount of DNP left in solution against NaCl concentration—was originally (2) determined in order to study the method of preparation as such. In this investigation it was found that the curve had a very characteristic appearance. The DNP molecule was also found to give different intrinsic viscosities and different sedimentation constants for different salt concentration of the solution (3).

From a DNP solution in 1–2 M NaCl buffered with phosphate to pH 6.3 (ion strength of buffer 0.01, for the fish liver series 0.05) samples of 1 or 2 ml were taken. Cold distilled water was added to each sample to give the desired NaCl molarity. In this way a series of dilutions with different total volumes was obtained.

The diluted solutions were shaken vigorously and were then allowed to stand in the cold for 20 hours (for the fish liver series 4 hours). The samples were then centrifuged at about 20,000 × g for 30 minutes. The supernatants were collected and the concentration of DNP still in solution was measured both by the nitrogen content (determined with micro Kjeldahl) and the UV absorption (determined in the Beckman spectrophotometer). The UV measurements were made between 230 and 400 mμ. The maximum absorption was obtained at 258–260 mμ for DNP from calf and ray fish but somewhat higher, or about 265 mμ, for DNP from the whiting. The maximum value has in all cases been corrected for background absorption using the values at 320–400 mμ (4). This correction was small for DNP from calf—the absorption at 370–400 mμ was less than 1 % of the maximum value. For the DNP from ray fish the absorption at 370–400 mμ was less than 2 %, but for the preparation from whiting it was much higher, between 5 and 10 % of the maximum value.

Since the DNP could never be completely dried, the NaCl concentration of the initial solution became lowered a few per cent and thus the salt content had to be redetermined. This was done for each series on the supernatants of the two solutions with the smallest DNP content (where no appreciable amount of nucleic acid was present) by igniting with sulphuric acid and ammonium carbonate and weighing the resulting sodium sulphate.

The values for the NaCl concentration were found to be accurate within ± 2 %. This uncertainty includes the effect of coprecipitation of NaCl by DNP.

The amount of sodium sulphate arising from the buffer was previously corrected for.

Desoxyribonucleoprotein (DNP) prepared with the use of solvents with different NaCl content

a. 1–2 M NaCl

DNP was prepared from calf thymus as described above. Three preparations were made, one using 1 M, one with 1.5 M and finally one with 2 M NaCl (all buffered as described above).

Precipitation curves were determined for all three preparations. Fig. 1 gives the curves for the preparations made with 2 M and 1.5 M NaCl. For the former concentration see also Fig. 3. No significant difference was found between that curve and the curves for preparations in 1 M (see earlier papers, 2, 3). Thus the straight line of dilution was followed to about 0.90 M where the precipitation first becomes evident. The first discontinuity point (overlooked in earlier work when starting from 1 M NaCl)

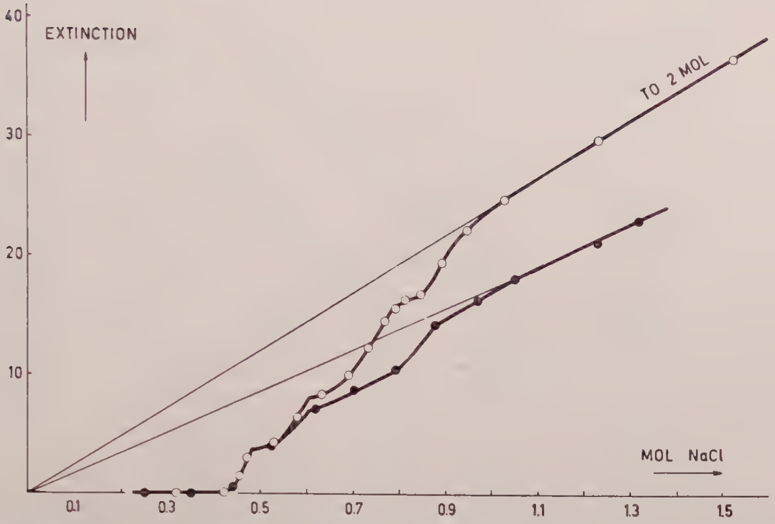


Fig. 1. Extinction at 260 mμ (1.00 cm cuvette) plotted against the NaCl content for a series of solutions which were prepared from a nucleoprotein dissolved in 2 M NaCl (unfilled circles) and in 1.5 M NaCl (filled circles) by dilution with distilled water. The precipitated part of the protein was centrifuged down and the extinction measured on the supernatant.
The original solutions were buffered with phosphate to pH 6.3 (ionic strength 0.01). The nucleoprotein was prepared from thymus according to MIRSKY and POLLISTER, i.e. in NaCl solutions 0.14 M and higher.

appeared at 0.78 M, the second at 0.60 M, and the third at 0.48 M, where the solubility decreases rapidly down to zero at about 0.40 M NaCl.

It must be stressed that whereas the typical discontinuities of the curves always appeared at the same NaCl concentrations, the corresponding DNP concentrations could vary considerably.

b. Distilled water

DNP prepared in 1 M NaCl + phosphate buffer was precipitated by dilution to 0.14 M. The precipitate was washed with and then redissolved in distilled water. The DNP was then reprecipitated, this time by adding solid salt to make the solution 0.14 M in NaCl. The precipitate was collected by centrifugation and then redissolved in 1 M NaCl and phosphate buffer. Not all the DNP precipitate from the distilled water solution could be brought back into solution in 1 M NaCl. The greater part, however, was dissolved and part of the precipitation curve determined. (See Fig. 2, curve *A*.) Complete precipitation occurred without any change from the normal.

A very different behaviour was observed for DNP prepared according to BERNSTEIN and MAZIA (5).

Using this method the ground thymus was first carefully washed with 0.9 % NaCl and thereafter extracted five times with distilled water, centrifuging after each extraction. The three last extracts which hold the bulk of the nucleoprotein were mixed and NaCl was added to 0.9 %. The precipitate was collected and redissolved in distilled water. The new solution was centrifuged for 60 minutes at 18,000 × g. NaCl was added to the clear supernatant to make it again 0.9 % and the precipitate was collected by centrifugation. This precipitate was redissolved and the cycle

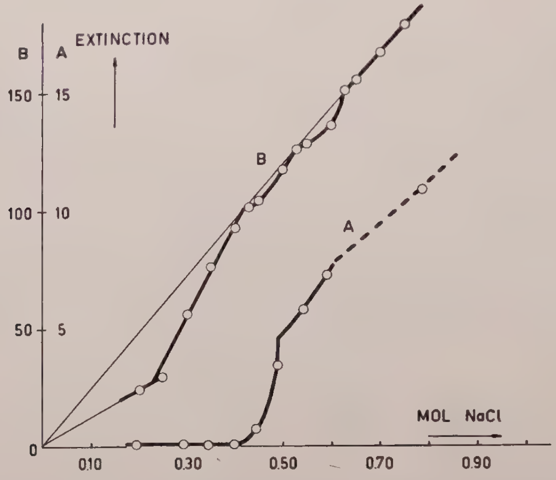


Fig. 2. Extinction at 260 mμ (1.00 cm cuvette) plotted against the NaCl content for two series of solutions which were prepared from nucleoprotein dissolved in 1 M NaCl (phosphate buffer pH 6.3, ionic strength 0.01).
The nucleoprotein giving curve *A* has been prepared from thymus according to MIRSKY and POLLISTER (NaCl solutions 0.14–1 M), and dissolved in distilled water once before determining the precipitation curve in the figure. The nucleoprotein giving curve *B* has been prepared from thymus according to BERNSTEIN and MAZIA (NaCl solutions 0.00–0.14 M) and dissolved in 1 M NaCl to determine the precipitation curve.

repeated three times in all. The last precipitate was dissolved in 1 M NaCl (buffered with phosphate to pH 6.3, ionic strength 0.01). For this solution the usual precipitation curve was determined. When the samples were diluted it was observed that the precipitate was more or less non-fibrous.

The precipitation curve (Fig. 2, curve *B*) differs in two respects from the usual type. First, the maximum precipitation is at a lower salt concentration and the whole curve is shifted in this direction. This fact may account for the difference in the solubility of DNP from sea urchin sperm found by MIRSKY and POLLISTER (1)—2 M NaCl necessary—and the solubility given in the curve published by BERNSTEIN and MAZIA for material from this source, for which some DNP is in solution at about 0.8 M. The second difference in the curve between DNP prepared in distilled water and that prepared in 1 M or higher is that the DNP from the former method possesses a soluble portion after maximum precipitation. This type of curve given by a non-fibrous form of DNP has been discussed by LUCK *et al.* (7).

The details of the curve were found to be reproducible. The slope of some parts of the curve is smaller than that of the line of dilution. The explanation of this is that each point is determined on a supernatant in equilibrium with a precipitate and that each sample giving this supernatant has been precipitated separately from the original DNP, i.e. 1 M NaCl solution.

The solubility of the non-fibrous protein is apparently higher than that of the fibrous.

Desoxyribonucleoprotein (DNP) prepared from liver

The DNP from liver prepared according to MIRSKY and POLLISTER was found to give the same precipitation curve as DNP from thymus (Fig. 3, unfilled circles).

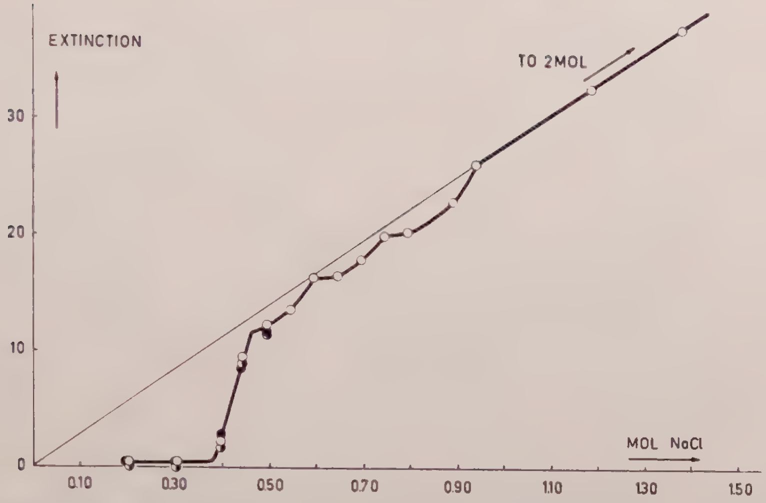


Fig. 3. Extinction at 260 mμ (cuvette 1.00 cm) plotted against NaCl content for a series of solutions which was prepared from nucleoprotein prepared according to MIRSKY and POLLISTER and dissolved in 2 M NaCl (phosphate buffer to pH 6.3, ionic strength 0.01). The dilutions were made with distilled water and the measurements carried out on the supernatants after the precipitation, if any, had been centrifuged down.
Unfilled circles—values on fresh prepared material. *Half-filled circles*—values on the same material, but making the dilutions one month later. *Filled circels*—values after the material had been treated with liver extract for 48 hours in 2 M NaCl + phosphate buffer (at + 4° C).

The DNP was of the fibrous type, which could be wound around a rod and which is insoluble at 0.30 M NaCl.

It was found, however, that the liver tissue DNP sometimes gave a precipitation curve showing a soluble portion even at low ionic strength and the precipitate itself was then also of a much less fibrous type and had to be collected by centrifugation during preparation.

The observation was made that the second type of DNP was obtained if during the first purification step the precipitate was allowed to stay for longer time in the 0.14 M NaCl mother solution.

A simple experiment was carried out which indicated the probable enzymatic character of the changes.

Part of the precipitation curve (Fig. 3, half-filled circles) of the DNP from liver, giving the curve in Fig. 3, was redetermined after the DNP solution in 2 M NaCl and phosphate buffer had been kept for one month in the cold. No change was observed. One hundred twenty-five g of a fresh liver was then sliced and washed with 100 ml 0.9 % NaCl. The extract was collected, part of it was made 2 M in NaCl and 2 ml of this were added to 30 ml of the one-month-old DNP in 2 M NaCl. The mixture was allowed to stand in the cold for 48 hours. Part of the precipitation curve was determined (Fig. 3, filled circles) using a solution of liver extract in 2 M NaCl treated completely in parallel with the DNP as a blank in the spectrophotometer. No changes were observed.

30 ml of the same one-month-old liver DNP was then precipitated with distilled water and 5 ml of the liver extract was added to the mother liquid (390 ml) with the precipitate in it. The mixture was allowed to stand in the cold for 48 hours. The preci-

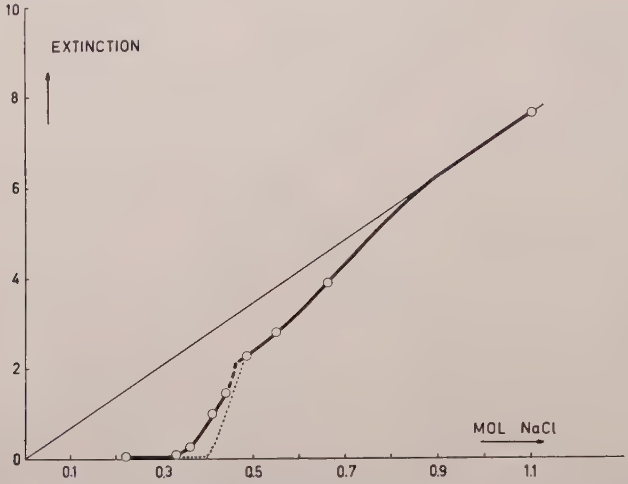


Fig. 4. Extinction at 260 mμ (cuvette 1.00 cm) plotted against NaCl content for the same nucleoprotein from liver as giving curves in Fig. 3.
The material has this time been treated as a precipitate in a 0.14 M NaCl solution with liver extract at + 4° C for 48 hours. The precipitate was redissolved in 1 M NaCl + phophate buffer to make the determination of the precipitation curve in the figure. The dotted line indicates the original form of the precipitation curve.

pitate was then collected, dissolved in 1 M NaCl (phosphate buffer pH 6.3, ionic strength 0.01) and this solution was kept in the cold for 24 hours, whereafter a part of the precipitation curve was determined (Fig. 4). The precipitate was less fibrous and the curve showed a significant difference between 0.3 and 0.5 M compared to the usual precipitation curve.

In a final experiment 10 ml of the liver DNP in 2 M NaCl was precipitated and the precipitate dissolved in distilled water. A few drops of the liver extract were added and the solution was allowed to stand in the cold for 48 hours.

Solid NaCl was added to the solution to 0.9 %. Some of the DNP precipitated but 35–45 % of the absorbing material stayed in solution.

The findings are in good agreement with the results of WEBB (8) on desoxypentosenuclease from calf thymus. He finds this enzyme to be active in distilled water, with optimal activity at 0.06 M NaCl and inhibited by NaCl solutions stronger than 0.2 M. The different behaviour between preparations of DNP from thymus and from liver may be due to differences in the amount of enzyme present in the different tissues.

Precipitation curves for desoxyribonucleoprotein (DNP) from different animals

DNP was prepared from liver from ray (*Raja batis*) and whiting (*Gadus merlangus*). In order to spare material it was found necessary to reprecipitate the DNP only three or four times and the precipitates were collected by centrifugation. The dilutions were made to 0.20 M NaCl for each reprecipitation. The precipitation curves, starting from 2 and 1.5 M NaCl respectively (buffered with phosphate to pH 6.3, ionic strength 0.05) are shown in Fig. 5 a and b.

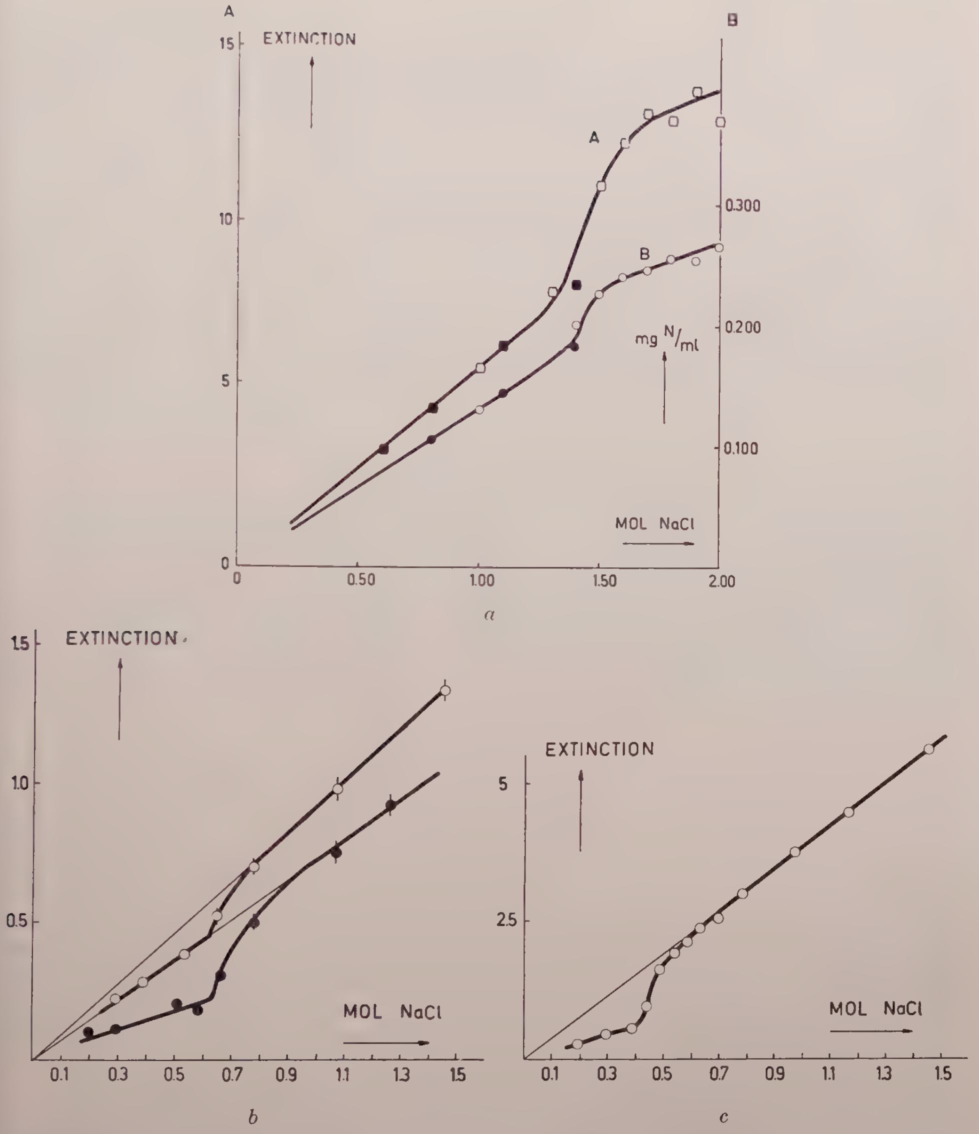


Fig. 5. Extinction at 260 mμ (265 for *b*) (1.00 cm cuvette) or nitrogen content plotted against the NaCl content for a series of solutions which were prepared from a nucleoprotein dissolved in 2 M or 1.5 M NaCl by dilution with distilled water. The precipitated part of the protein was centrifuged down and the determinations made on the supernatant. The original solutions were buffered with phosphate to pH 6.3 (ionic strength 0.05). The nucleoprotein was prepared according to Mirsky and Pollister, i.e. in NaCl solutions 0.14 M and higher.

a. Nucleoprotein from liver of ray (*Raja batis*). The curve is from one of two very similar preparations reprecipitated 3 and 4 times. Filled and unfilled symbols refer to separate centrifugations of the diluted samples. *b*. Nucleoprotein from liver of whiting (*Gadus merlangus*). The upper curve has been reprecipitated 4, the lower 3 times. The vertical lines indicate the error given to each point due to the uncertainty of the correction for background absorption. *c*. Nucleoprotein from liver of calf. The protein was reprecipitated 3 times.

The curve shown for the ray fish preparation corresponds to one of two such preparations, both of which gave very similar curves. Fig. 5 c shows the precipitation curve for DNP from liver precipitated from 1.5 M NaCl (phosphate buffer pH 6.3, ionic strength 0.05). This last preparation was purposely made in such a way that its first precipitation was left for 5 hours in contact with the mother liquid, to give the enzyme time to work. The preparations from the fishes showed a high portion of UV absorbing material which was soluble after maximum precipitation and the aim was to make all preparations as like one another as possible. All work was done in the cold room. The possibility that the enzymes from fishes from northern waters are more active in the cold than enzymes from warm-blooded animals is perhaps not an unlikely one. The spawn of whiting will for instance develop even in sea water at − 1° C (9). Differences between the enzymes may of course also exist in sensitivity to salt content and pH. The latter factor is being investigated.

Discussion and conclusion

It may be stated that there will be no significant changes in the precipitation curve for DNP extracted from calf thymus with 1 M, 1.5 M, or 2 M NaCl. Changes will occur only if the DNP precipitate is allowed to stand in contact for a long time with its first mother liquid at low NaCl strength or if the preparation is made in distilled water. The first-mentioned changes are very small. Since the relative activity of the enzymes of the fishes is not known, there is a possible source of error when comparing different preparations. In other words, the part of the precipitation curve indicating increase in maximum precipitation will be shifted to somewhat lower salt concentrations compared with the fibrous DNP of calf liver. The two series for each fish species were made, however, with DNP precipitated three and four times respectively and no difference was found for the point at which maximum increase in precipitation occurred for the two preparations. This gives some indication that the error will not be very large if these curves are compared with a DNP from calf liver, where the precipitate had been in contact 5 hours with the first mother liquid.

On the other hand, the DNP preparations from ray, whiting and calf liver show a large difference in precipitation behaviour. The maximum increase in precipitation falls at about 1.45 M NaCl as seen in Fig. 5 a, b, and c for DNP from ray liver, for DNP from whiting liver at about 0.65 M NaCl, and for DNP from calf liver at about 0.45 M NaCl. These findings may be correlated to the osmotic pressure of the body fluids, a direct measure of which is their freezing point depression.

The freezing point depression of the blood serum of the ray fish genus is given in the literature as 1.92, 1.93 (10), 1.89, 1.96 (11) degrees. The values are given for species of ray fishes other than the one used here but they live in sea water of the same salinity. The ray fish from which the liver has been used in this study was taken in waters with 3.3–3.4 % salts.

The freezing point depression for ray will be given as 1.92 degrees in the following.

For the whiting the freezing point depression of blood is listed at 0.860 degrees (12). The value for serum would then fall in the region of 0.75–0.82 (12, 13) degrees if compared with values given for other fishes of the same genus and considering the salt content (3 %) of the sea water where the whiting was taken.

For cattle, references give the freezing point depression values 0.585 (11), 0.598, 0.611 (12) degrees (mean value of series with 0.560 as lowest and 0.633 as highest value). The value 0.60 degrees will be used in the following.

If the freezing point depression for serum of each animal is divided by the salt concentration at which the precipitation curve shows the steepest slope we get the following quotients:

ray fish	$1.92/1.45 = 1.32$
whiting	$0.78/0.65 = 1.20$
calf	$0.60/0.45 = 1.33.$

If reasonable uncertainty is given to each value, all three quotients can be expressed as 1.25 ± 0.15. It can thus be stated that the DNP prepared according to MIRSKY and POLLISTER will show a precipitation behaviour which is in direct correlation to the osmotic pressure of the tissue medium of the species in question.

Even if this may be taken as a first correlation of any of the characteristics of DNP *in vivo* with DNP *in vitro*, it must be emphasized that the effect is probably due to the protein moity of the DNP and no conclusion can therefore be drawn from this correlation as to whether nucleic acid becomes associated with the protein in the cell or in the test tube as a result of preparation. It is, however, very tempting to point out that the insolubility of the DNP complex as a function of tissue environment seems to be a very healthy thing for the animals. This is especially true of the elasmobranch where the nucleoprotein (if existing) would be dangerously near to being brought into solution if the elasmobranch DNP had the same solubility as that of mammals.

Summary

Desoxyribonucleoprotein was prepared (according to the method of MIRSKY and POLLISTER, slightly modified) from animals with different freezing point depressions in their body fluids.

The nucleoprotein was precipitated by diluting from 2 M to lower NaCl concentrations. The maximum increase in precipitation of nucleoprotein for a small decrease in NaCl content was found to occur at different NaCl concentrations for different animals. There is a direct proportionality between the salt concentration in question and the freezing point depression of the body fluids of the animal.

ACKNOWLEDGEMENTS

The author wishes to thank Professor A. TISELIUS for his kind interest and helpful criticism. It has also been very helpful indeed to be able to discuss this paper with Dr. D. KUPKE (Stanford University).

For their kind help in delivering the necessary fish material, the author is indebted to fil. kand. L. HANNERZ at the marine station of the Royal Swedish Academy of Science, Fiskebäckskil, and to Mr. K. FLOBERG, Gothenburg.

The expenses connected with this work have been defrayed by grants from the Rockefeller and Wallenberg Foundations.

Institute of Biochemistry, University of Uppsala, Sweden.

REFERENCES

1. A. E. Mirsky and A. W. Pollister, Proc. Nat. Acad. Sci. U.S. *28*, 344 (1942).
2. G. Frick, Biochim. Biophys. Acta *3*, 103 (1949).
3. G. Frick, Biochim. Biophys. Acta *8*, 202 (1952).
4. W. Heller und E. Vassy, J. Chem. Phys. *14*, 565 (1946).
5. M. H. Bernstein and D. Mazia, Biochim. Biophys. Acta *10*, 600 (1953).
6. M. H. Bernstein and D. Mazia, Biochim. Biophys. Acta *11*, 59 (1953).
7. J. M. Luck, D. W. Kupke, A. Rhein and M. Hurd, J. Biol. Chem. *205*, 235 (1953).
8. M. Webb, Exptl. Cell Research *5*, 27 (1953).
9. Tabulae Biologicae *6*, 583 (1930).
10. H. W. Smith, Biol. Rev. *11*, 49 (1936).
11. L. V. Heilbrunn, An Outline of General Physiology, W. B. Saunders Co., Philadelphia, 2nd ed. 1947, p. 113–114.
12. Tabulae Biologicae *1*, 498–503 (1925).
13. A. B. Macallum, Physiol. Rev. *6*, 316 (1926).

Tryckt den 25 augusti 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 48

Communicated 14 April 1954 by ARNE FREDGA

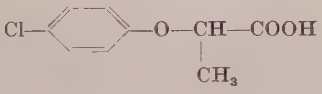
Stereochemical studies on plant growth regulators. IX[1]

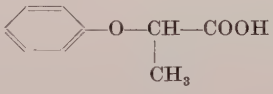
The enantiomorphic α-(4-chlorophenoxy)propionic acids and their configuration

By MAGNUS MATELL

With 3 figures in the text

Chloro-substituted α-aryloxyalkylcarboxylic acids are often found to be very efficient plant growth regulators. Several optically active compounds of this type have been investigated. The results have recently been summarised by ÅBERG (1) and by MATELL (2). From stereochemical as well as from plant physiological points of view it now seemed important to extend these investigations to α-(4-chlorophenoxy)propionic acid (I). The racemic acid is known to possess fairly strong auxin activity but the optically active forms do not seem to have been described previously.


I


II

The racemic acid was resolved using brucine to yield the (−)-form and (−)-β-phenyl*iso*propylamine for the (+)-form. The rotatory power was determined in different solvents. As seen from Table 1 the optical activity data give a de-

Table 1. $[\alpha]_D$ for (+)-α-(4-chlorophenoxy)propionic acid (I) and (+)-α-(3.4-dichlorophenoxy)propionic acid ($3.4Cl_2POP$) in different solvents.

Solvent	$[\alpha]_D$	
	I	$3.4Cl_2POP$
Acetone	+57.4°	+54.8°
Ethanol	+41.0°	+39.1°
Chloroform	+23.4°	+23.9°
Water (Na-salt)	+17.9°	+14.6°
Benzene	−11.1°	+ 3.5°

---

[1] Part VIII: Arkiv Kemi *6*, 375 (1953).

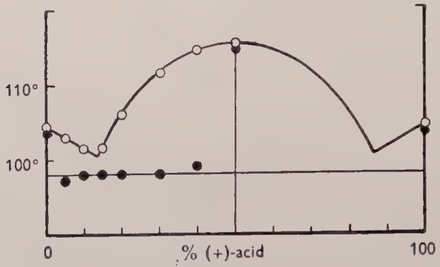


Fig. 1. (+)- and (−)-α-(4-chlorophenoxy)propionic acid.

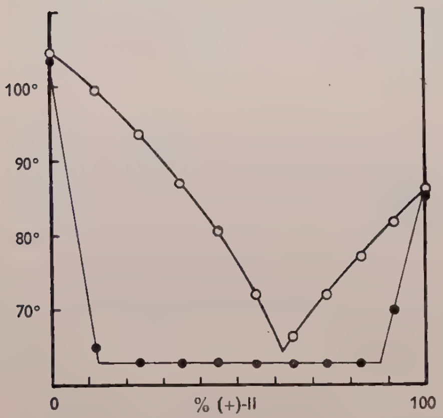


Fig. 2. (+)-α-(4-chlorophenoxy)propionic acid (I) and (+)-α-phenoxypropionic acid (II).

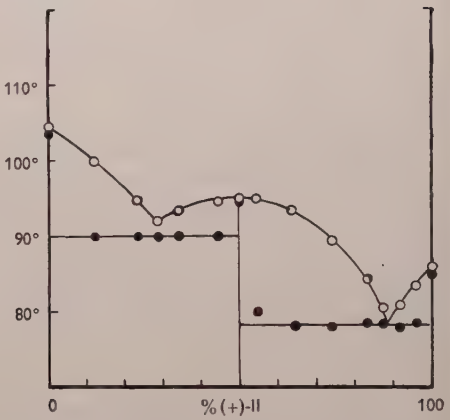


Fig. 3. (−)-α-(4-chlorophenoxy)propionic acid (I) and (+)-α-phenoxypropionic acid (II).

creasing series when the solvents are arranged in the same order as for (+)-α-(3.4-dichlorophenoxy)propionic acid (3.4Cl_2POP). A similar regularity can be demonstrated for several other acids of the same type (2) and this is strong evidence for identical configuration. Considering previous work on the configuration of similar compounds the (+)-form of I should belong to the D-series (2).

A more conclusive proof was obtained using the method of quasi-racemates (3). As seen from Fig. 1 the racemic form of the acid I is a true racemic compound. The melting point of the racemate is more than 10° higher than that of the antipodes, on account of which the racemate formation tendency may be characterized as moderate. Therefore the chances of obtaining quasi-racemates were considered to be rather small except for structurally very closely related compounds. Among compounds available for a direct comparison the 2.4-dichloro- and 3.4-dichlorophenoxypropionic acids seemed most promising. However, preliminary experiments using the contact method of KOFLER (4) were without success. As regards structural conditions the unsubstituted phenoxypropionic acid II should be less prone to form a quasi-racemate with the 4-chloro-substituted acid I. On the other side α-phenoxypropionic acid has a considerably larger tendency for racemate formation (5) than the dichloro-substituted acids (6). In this case the latter fact seems to be the determining factor as the preliminary experiments revealed the formation of a quasi-racemic compound between the acids I and II with opposite directions of optical rotation.

To get a more detailed picture of the phase systems in question, melting point diagrams were determined in the usual manner (2). As seen from Fig. 3 the acids with opposite directions of rotation form a molecular compound characterized by the well-developed middle branch of the curve. The acids with the same direction of rotation give a simple eutectic diagram (Fig. 2) and the racemic nature of the molecular compound is thus established. Consequently the acids I and II which have the same direction of rotation also have the same configuration. The configuration of α-phenoxypropionic acid has already been determined; the (+)-form belongs to the D-series (2, 5). The same is then true for (+)-α-(4-chlorophenoxy)propionic acid too.

The plant growth regulating properties of the optically active forms of I have been studied by Dr. BÖRJE ÅBERG who has kindly put some data at the author's disposal. Compared with the (+)-forms of II and α-(2.4-dichlorophenoxy)propionic acid the auxin-activity of (+)-I is intermediate. The (−)-form is about 100 times less active than the (+)-form. The physiological experiments will be described in full detail by Dr. ÅBERG in a separate paper.

It has previously been stated that optically active α-aryloxyalkylcarboxylic acids which have greater auxin-activity than their corresponding antipodes, belong to the D-series (2). The validity of this statement is thus further verified by the experiments presented here.

Experimental

α-(4-Chlorophenoxy)propionic acid (I).—The racemic acid was prepared in the usual manner by refluxing equimolecular parts of sodium 4-chlorophenolate and ethyl α-bromopropionate in abs. ethanol, followed by hydrolysis with aqueous sodium hydroxide. The crude acid was recrystallised from 50 % formic acid. Yield of pure acid 70 %, m.p. 114.5–115.5° in good agreement with the literature (7).

80.31 mg acid: 8.06 ml 0.0495-*N* NaOH
$C_9H_9O_3Cl$ calc. equiv. wt. 200.6
found ,, ,, 201.1

Preliminary experiments on resolution.—Of the common alkaloids only brucine gave an appreciable resolution; from the salt a laevorotatory acid was isolated. Cinchonine, quinidine and strychnine yielded crystalline salts from which nearly quite inactive products were obtained. Good results were recorded with optically active α-phenethylamine and β-phenyl*iso*propylamine. The latter base and brucine were used for the final resolution.

(−)-α-(4-Chlorophenoxy)propionic acid.—Inactive acid (30.1 g, 0.15 mole) and an equivalent amount of brucine (38.6 g) were dissolved in a hot mixture of ethanol (200 ml) and water (300 ml). On standing at room temperature the salt crystallised and was collected after a day. The salt was recrystallised several times as seen from Table 2; the rotatory power of the acid was measured in abs. ethanol.

Table 2. Recrystallisation of the brucine salt of α-(4-chlorophenoxy)-propionic acid.

Cryst. No.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D$ of the acid
	Water	Ethanol		
1	200	300	65.9	–
2	300	300	39.1	−35.7°
3	250	250	35.1	−38.9°
4	250	250	31.7	−38.3°
5	200	200	29.4	−39.4°

28.4 g of the pure salt was decomposed with dilute hydrochloric acid yielding 8.9 g crude acid. It was recrystallised first from 50 % formic acid and then from petroleum (b.p. 60–80°). The pure (−)-acid (7.2 g) melted at 103.5–104.5°.

76.58 mg (−)-acid: 8.18_5 ml 0.0464-*N* NaOH
$C_9H_9O_3Cl$ calc. equiv. wt. 200.6
found ,, ,, 201.6
0.2022 g dissolved in abs. *ethanol* to 20.06 ml: $2\alpha_D^{25} = -0.808°$. $[\alpha]_D^{25} = -40.1°$; $[M]_D^{25} = -80.4°$.

(+)-α-*(4-Chlorophenoxy)propionic acid.*—From the mother liquors 1 and 2 (Table 2) an acid with $[\alpha]_D = +28°$ was isolated. This acid (15.1 g, 0.075 mole) and an equivalent amount of (−)-β-phenyl*iso*propylamine (10.1 g) were dissolved in hot 50 % ethanol (150 ml). On adding water (175 ml) the salt crystallised and was collected after standing some hours in a refrigerator. The salt was recrystallised according to Table 3. The rotatory power of the acid was measured in abs. ethanol.

Table 3. Recrystallisation of the (−)-β-phenyl*iso*propylamine salt of α-(4-chlorophenoxy)propionic acid.

Cryst. No.	Solvent (ml)		Yield of salt (g)	$[\alpha]_D$ of the acid
	Water	Ethanol		
1	75	250	17.8	+39.1°
2	75	200	14.2	+39.1°
3	50	150	11.7	+39.8°

The acid was isolated from 11.2 g salt from the last fraction. The crude acid (6.9 g) was recrystallised first from 50 % formic acid and then from petroleum (b.p. 60–80°) yielding 4.5 g of the pure (+)-acid, m.p. 103.5–104.7°.

81.76 mg (+)-acid: 8.76_5 ml. 0.0464-*N* NaOH
$C_9H_9O_3Cl$ calc. equiv. wt. 200.6
found ,, ,, 201.0

Table 4. (+)- and (−)-I

% (+)-I	M.p.
0.0	103.5–104.5°
5.0	97 –103°
10.1	98 –101.5°
14.9	98 –101.5°
20.0	98 –106°
30.0	98 –111.5°
39.8	99 –114.5°
50.0	114.5–115.5°
100.0	103.5–104.5°

Table 5. (−)-I and (+)-II

% (+)-II	M.p.
0.0	103.5–104.5°
12.1	90 –100°
23.3	90 – 95°
28.6	90 – 92°
34.1	90 – 93.5°
44.5	91 – 94.5°
49.9	94.5– 95°
54.4	80 – 95°
64.4	78 – 93.5°
74.0	78 – 89.5°
83.0	78.5– 84.5°
87.2	78.5– 80.5°
91.5	78 – 81°
95.7	78.5– 83.5°
100.0	85 – 86°

Table 6. (+)-I and (+)-II

% (+)-II	M.p.
0.0	103.5–104.5°
11.8	65 – 99.5°
23.5	63 – 93.5°
34.7	63 – 87°
44.7	63 – 80.5°
54.7	63 – 72°
64.4	63 – 66.5°
73.8	63 – 72°
82.8	63 – 77°
91.6	70 – 81.5°
100.0	85 – 86°

0.1753 g dissolved in abs. *ethanol* to 20.06 ml: $2\alpha_D^{25} = 0.716°$. $[\alpha]_D^{25} = +41.0°$; $[M]_D^{25} = +82.2°$.

0.1816 g dissolved in *acetone* to 20.06 ml: $2\alpha_D^{25} = +1.039°$. $[\alpha]_D^{25} = +57.4°$; $[M]_D^{25} = +115.1°$.

0.2028 g dissolved in *chloroform* to 20.06 ml: $2\alpha_D^{25} = +0.474°$. $[\alpha]_D^{25} = +23.4°$; $[M]_D^{25} = +47.0°$.

0.2101 g dissolved in *benzene* to 20.06 ml: $2\alpha_D^{25} = -0.232°$. $[\alpha]_D^{25} = -11.1°$; $[M]_D^{25} = -22.2°$.

0.1779 g neutralized with *aqueous NaOH* and made up to 20.06 ml: $2\alpha_D^{25} = +0.318°$. $[\alpha]_D^{25} = +17.9°$; $[M]_D^{25} = +36.0°$.

Melting point diagrams.—Weighed quantities of the components were dissolved in a few drops of pure acetone which was evaporated. The residue was powdered and dried. All melting points were determined using a hot stage microscope according to KOFLER (4). The results are found in Tables 4–6 and Figs. 1–3 where the composition is given in mole-%. Contact preparations were studied with the same apparatus.

The author is indebted to Mr. A. AAMISEPP for technical assistance. The financial support of the *Swedish Natural Science Research Council* is gratefully acknowledged.

Summary

α-(4-Chlorophenoxy)propionic acid has been resolved into optical antipodes. The rotatory power of the active acid has been determined in different solvents.

By means of the method of quasi-racemates it has been shown that the (+)-form has the D-configuration. The auxin activity of the D-form is about 100 times greater than that of the (−)-form which is in agreement with previous statements on the stereo-chemical specificity of plant growth regulators of the α-aryloxyalkylcarboxylic acid type.

Department of Organic Chemistry, Royal Agricultural College, Uppsala, April 1954.

REFERENCES

1. B. Åberg, Ann. Agr. Coll. Swed. *20*, 241 (1953).
2. M. Matell, Ibid. *20*, 205 (1953).
3. A. Fredga, The Svedberg 1884 $\frac{30}{8}$ 1944, Uppsala 1944, p. 261.
4. L. Kofler and A. Kofler, Mikro-Methoden, Innsbruck 1948.
5. A. Fredga and M. Matell, Arkiv Kemi *4*, 325 (1952).
6. M. Matell, Ibid. *5*, 341 (1953).
7. D. Osborne, R. L. Wain and R. Walker, J. Hort. Sci. *27*, 44 (1952).

Tryckt den 25 augusti 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 49

Read 12 May 1954

Chromatography of proteins on calcium phosphate columns

By Arne Tiselius

With 2 figures in the text

During recent years several successful attempts have been made to extend the applications of chromatography to the separation of proteins. Columns of ionic exchange resins (IRC 50) have proved very useful in the separation of proteins of small molecular weights (e.g. cytochrome C, ribonuclease, lysozyme) (1–6) but also hemoglobins have been chromatographed on such columns (7). Partition chromatography has proved possible with some proteins (e.g. ribonuclease, insulin) as shown by Herbert and Pinsent (8) and Martin and Porter (9, 10). Adsorption chromatography of proteins has proved to be rather difficult, and several attempts in this direction have met with only partial success. This is also true of chromatographic separation of proteins based upon the phenomena of "salting out" or "salt-promoted" adsorption studied by the author (11), which, however, has found some interesting applications with proteins of very large particle size, see for example (12). It appears that the attainment of adsorption equilibrium generally is too slow for column operation and no doubt many adsorbents bind proteins irreversibly, possibly on account of surface denaturation.

On the other hand the usefulness of adsorbents in preparation work by "batch operation" with proteins, particularly enzymes, has been amply proved (13) and has demonstrated very striking specific differences in adsorption affinities which make it very tempting to look again into the possibilities of working out a chromatographic procedure for proteins based upon specific differences in adsorption.

The present study is a continuation of some earlier work in this laboratory to utilize precipitated calcium phosphate as a column material for the separation of proteins (14). In that investigation the calcium phosphate was mixed with five parts (by weight) of Super-Cel to facilitate the filtration. In the present work it was found possible to prepare the calcium phosphate in a form which makes it unnecessary to use any filter aid. This is believed to be an advantage as Super-Cel tends to adsorb some proteins under the conditions of our experiments, and thus complicates the procedure. Precipitated calcium phosphate appears to be an adsorbent which is particularly well suited for work of this kind, and there are some earlier investigations of its use for chromatography of proteins and enzymes (15, 16). The precipitate, although of crystalline structure as shown by X-ray diagrams, is a gel, very rich in water.[1] We have the impression that strongly hydrophilic adsorbents should be preferred in protein chromatography as their tendency to cause surface denaturation appears to be less than with substances of a more hydrophobic character. Besides, the gel-like character would tend to make the surface more accessible even to large protein molecules.

---

[1] The author is indebted to Prof. G. Hägg for valuable aid in taking X-ray powder diagrams of the calcium phosphate preparations.

Preparation of the adsorbent and the columns

After several experiments we came to the conclusion that the best material was obtained by precipitation at room temperature of calcium chloride (0.5 M) with an excess of disodium phosphate (0.5 M), adding the precipitant gradually under stirring. The precipitate is washed repeatedly with disodium phosphate of the concentration to be used in the column. The precipitate is stored in equilibrium with this solution and columns are packed simply by pouring the suspension into a glass tube with a plug of glass wool in the outlet, finally applying a pressure of about 0.4 atm. to compress the material. A column of 80 mm length and 15 mm diam. will let through about 1–3 ml an hour without external pressure, which is a suitable rate of filtration to give sharp and even zones. These columns are very easy to handle, the material keeps so well together that they may even be turned upside down, and it is very easy to change the supernatant elution medium without damaging the top layer.

Procedure

As the experiments described in this paper had as their main purpose to study the possibilities of using calcium phosphate columns for protein chromatography it was thought advantageous first to try coloured proteins and thus follow the separation by direct visual observation. Thus phycoerythrin and phycocyan from *Ceramium rubrum* were used first, as these are quite well-defined, stable and strongly coloured proteins. For further details about the preparation and properties of these proteins see ref. (14). It should be noted that their mol. wts are 290,000 and 270,000 resp. at neutral reaction and the isoelectric points pH = 4.25 and pH = 4.85 respectively. Some experiments were also made with a sample of carbon monoxide hemoglobin. Since visual observation was found to be so valuable, attempts were made to observe directly also the chromatography of colourless proteins. This could be arranged by photographing the tube in reflected ultraviolet light of a wave length in the neighbourhood of the typical absorption maximum of proteins at 260–280 mμ. For this purpose a tube of fused, clear quartz was used for the column, with two Philips quartz mercury lamps Type 93110 E as illumination, placed at a distance of about 60 cm in front of the tube at an oblique vertical angle to avoid reflections from the tube wall. A camera with a quartz lens of 20 cm focal distance[1] and with quartz light filters containing chlorine and bromine gave an image essentially only in the ultraviolet, if non-orthochromatic plates were used (for example Process plates). It proved possible to obtain in this way rather satisfactory pictures of the chromatography of for example serum albumin, egg albumin, Helix hemocyanin and tobacco mosaic virus (see Fig. 2). A similar method has been used with transmittant light by SVEDBERG (17) and by the author (18, 19) in early work on ultracentrifugation and electrophoresis of proteins.

---

[1] A longer focal distance would probably be preferable to avoid parallactic effects, which however are hardly significant in these experiments.

A number of experiments were also run in a fraction collector, generally with 60 min. interval between the shifting of the tubes. The proteins were dissolved in phosphate buffer solution, mostly 0.005 M or 0.01 M Na_2HPO_4, and the column was washed with the same medium. Elution was performed by applying Na_2HPO_4 solutions of successively higher concentrations. In experiments with the fraction collector a simple gradient elution arrangement was used (20), giving automatically a slowly rising concentration of the eluant, which was recorded with a Micromax conductivity recorder with the conductivity cell placed in the mixing chamber. Analyses for protein in the collected samples were made by measuring the ultraviolet absorption in a Beckman DU spectrophotometer.

Protein preparations used

R-phycoerythrin and R-phycocyan: These proteins were prepared according to Kylin from aqueous extracts of Ceramium rubrum (see ref. 14 and 19). No attempt was made to separate the proteins from each other prior to the chromatographic experiments.

Human serum albumin: This was a commercial dried preparation (Kärnbolaget, Stockholm, lot n:o Ra H 16).

Egg albumin: prepared in this laboratory, three times recrystallized and freeze-dried.

Carbon monoxide hemoglobin (bovine): also prepared in this laboratory (see ref. 19).

Helix pomatia hemocyanin: Dialysed whole blood of the snail was used, without further purification.

Tobacco mosaic virus: was prepared according to Bawden and Pirie (21).

Results

Most orientation experiments were made with a mixture of phycoerythrin and phycocyan, as obtained by fractional precipitation with ammonium sulphate of aqueous extracts of Ceramium. These experiments served as a guide in finding the suitable calcium phosphate preparation, which would give even zones and a minimum of tailing. Even with the best columns it was found impossible to elute the proteins without excessive tailing under conditions giving strong rentention (Rf values below 0.5–0.6), in agreement with earlier observations by Swingle and the author (14). Thus elution of different components in a constant medium would require rather long columns. The elution effect is chiefly determined by the concentration of the eluant phosphate buffer, and it was soon found that each of the proteins change their Rf values from zero to about 1.0 in a rather narrow phosphate concentration interval. This is illustrated in Fig. 1, showing the separation of phycoerythrin and phycocyan by elution in two steps: 1. by 0.02 M Na_2HPO_4, 2. by 0.04 M Na_2HPO_4. It is seen that the phycocyan does not move at the lower phosphate concentration (except for a small initial migration when it is still accompanied by phycoerythrin which reduces the adsorption by displacement), but will migrate as a sharp zone in 0.04 M phosphate. This high sensitivity to the eluant concentration appears to be typical of proteins, and should be expected on theoretical grounds in any system where the adsorbed substances are polyvalent ions. Similar observations on the

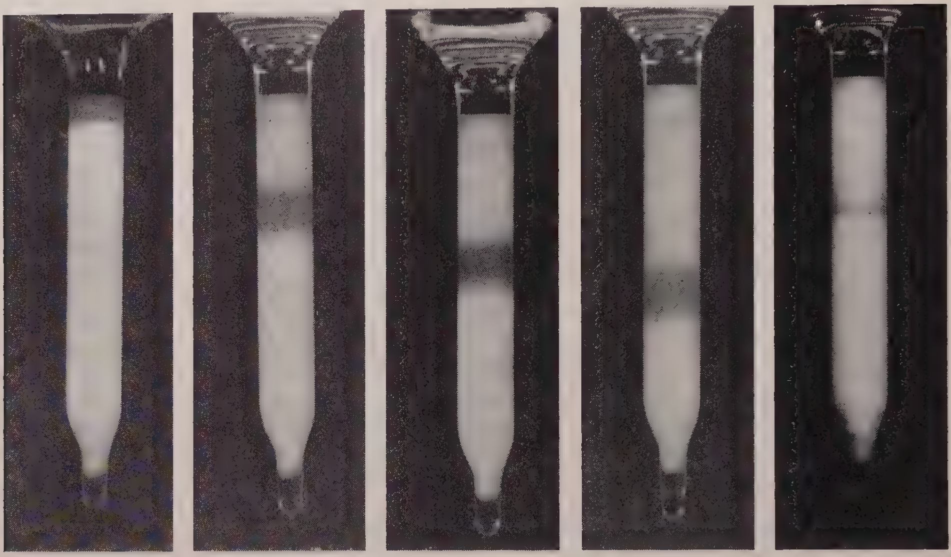


Fig. 1. (1–5) Chromatographic separation of R-phycoerythrin and R-phycocyan on a calcium phosphate column, equilibrated with 0.02 M Na_2HPO_4. Length of column 75 mm. cross section area 0.66 cm², volume 5.0 cm³. The sample was 1.0 ml of a mixture of R-phycoerythrin and R-phycocyan, total protein concentration 0.6 %, in 0.02 M Na_2HPO_4. Elution: 1. With 0.02 M Na_2HPO_4, exposures 1–4. The phycoerythrin zone is migrating, the phycocyan is stationary. 2. With 0.04 M Na_2HPO_4, exposure 5. The phycoerythrin has left the column, the phycocyan is now migrating.

pH-sensitivity in the elution of proteins from ionic exchange resins have been made by Fromageot (22). Evidently these phenomena may be made use of for a very efficient form of protein chromatography based upon the use of eluting solutions of successively increasing concentrations. Effects of pH and concentration of neutral salts (e.g. NaCl) offer further possibilities in varying the conditions of the experiment to obtain the best separation.

Table 1.

Protein	Elution range
R-Phycoerythrin	0.005–0.008 M Na_2HPO_4
R-Phycocyan	0.030–0.040 M Na_2HPO_4
Egg albumin: Component I . .	0.005–0.007 M Na_2HPO_4
,, ,, Component II . .	0.010–0.012 M Na_2HPO_4
Serum albumin (human)	0.050–0.070 M Na_2HPO_4
CO-Hemoglobin (bovine)	0.040–0.050 M Na_2HPO_4
Helix Hemocyanin	0.040–0.050 M phosphate buffer pH = 6.7 + 0.2 M NaCl
» »	0.008–0.010 M phosphate buffer pH = 6.7
Tobaco mosaic virus	0.100–0.120 M phosphate buffer pH = 6.7

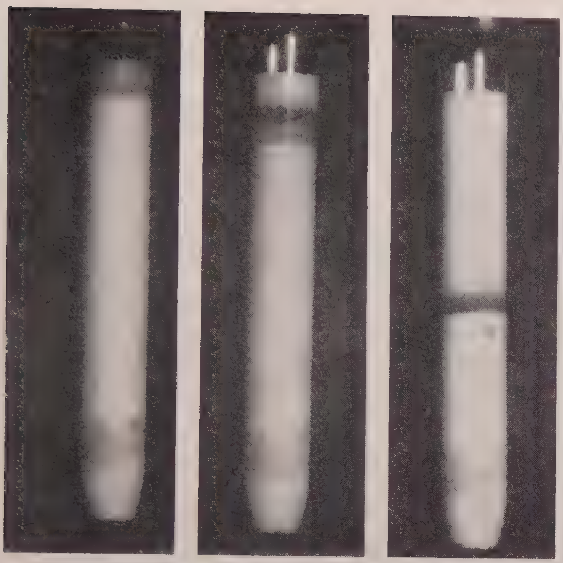


Fig. 2. (1–3) Chromatography of serum albumin on a calcium phosphate column, equilibrated with 0.01 M Na_2HPO_4. Quartz tube. Length of column 95 mm, cross section area 1.24 cm², volume 11.8 cm³. The sample was 2 ml 1 % serum albumin in 0.01 M Na_2HPO_4. Photographs taken in ultraviolet light with chlorine and bromine filters. Exposure 1 shows the serum albumin zone at the top, before elution has started. Exposure 2: elution with 0.20 M Na_2HPO_4. The buffer front is overtaking the protein front. Exposure 3: the same elution proceeding with the protein zone concentrated at the gradient.

Table 1 gives the approximate concentrations at which a number of proteins are eluted from calcium phosphate and serve as an illustration of the marked specificity. The values were obtained by observation on columns either visually or—with colourless proteins—by ultraviolet photography.

Fig. 2 shows the elution of a sample of human serum albumin (2 ml 1 % solution) on a calcium phosphate column, as obtained by photography in the short-wave ultraviolet. In this case an unnecessary high phosphate concentration has been used for the elution, giving rise to a very sharp zone. Actually, a less steep gradient showed the presence of two or possibly three components in this particular sample.

In several cases the proteins investigated showed more than one component and rechromatography of the separated components confirmed the reality of the separation. Thus several fractions of the Ceramium extract showed the presence of another red component which was eluted at a higher phosphate concentration (about 0.02 M) and also a faint blue-violet zone more strongly adsorbed than the main phycocyan component. It was also observed that samples which were partially denatured invariably gave a trace of a coloured zone which could be eluted only at very high concentration (0.2 M phosphate). It has not been possible to examine the various fractions obtained in this preliminary investigation, where the purpose has been to obtain a general idea of the possibilities of the method with a number of representative proteins, but such work is in progress.

The chromatographic fractions obtained in the experiments with hemocyanin and tobacco mosaic virus were further identified by their strong light scattering and—in case of the virus solutions—by electron microscopy.

Discussion

The experiments show that by using suitably prepared columns of calcium phosphate it is possible to perform chromatographic analysis of proteins in the molecular weight range from egg albumin to tobacco mosaic virus. The separation depends chiefly on the proteins possessing a markedly narrow and specific range of elution with regard to the concentration of the phosphate buffer solutions used as eluants. Differences in Rf values during the migration through the column contribute to the separation but the effects of the differences in eluant concentrations dominate. Thus the procedure differs somewhat from typical chromatography in a constant medium where Rf differences are decisive. As a consequence of this it has been found practical to localize the fractions obtained in a fraction collector rather by their concentration with regard to the buffer solution than with reference to the volume which has passed through the column.

The zones obtained were generally very sharp and even, actually better than in many experiments in ordinary chromatography. The quantities used were of the order of about 10 mg protein per 10 g of wet column material. It is possible to use very large columns, and it is easy to regenerate the column after an experiment. Thus the method appears promising also for preparative purposes. It would seem more practical, however, in such cases to use the chromatographic observations as a guide for batch adsorption - elution operations, particularly with proteins which show marked differences in their elution range. Thus (cf. Table 1 above) phycoerythrin and phycocyan are easily separated in quantities by adsorption at a concentration of 0.02 M Na_2HPO_4 (where only phycocyan is adsorbed), washing the calcium phosphate with the same solution and subsequently eluting with 0.04 M Na_2HPO_4.

It appears particularly interesting that proteins of even very high molecular weight like hemocyanin and tobacco mosaic virus lend themselves easily to chromatographic analysis by the procedure described.

The author is indebted to Mr. L. HAGDAHL for very active and valuable help in many of the experiments. The Ceramium material was provided by Dr. GUNNAR GUSTAFSON of the Kristineberg Zoological Station, which is gratefully acknowledged. The investigation has been financially supported by grants from the Rockefeller and Wallenberg Foundations.

Institute of Biochemistry, University of Uppsala, Sweden.

REFERENCES

1. S. PALEUS and J. B. NEILANDS, Acta Chem. Scand. *4*, 1204, 1950.
2. C. H. W. HIRS, W. H. STEIN, and S. MOORE, J. Am. Chem. Soc. *73*, 1893, 1951.
3. H. H. TALLAN and W. H. STEIN, J. Am. Chem. Soc. *73*, 2976, 1951.
4. C. H. W. HIRS, S. MOORE, and W. H. STEIN, J. Biol. Chem. *200*, 493, 1953.
5. H. H. TALLAN and W. H. STEIN, J. Biol. Chem. *200*, 507, 1953.
6. —— Federation Proceedings, Vol. 11, No. 1, 1952.
7. N. K. BOARDMAN and S. M. PARTRIDGE, Nature *171*, 208, 1953.
8. D. HERBERT and J. PINSENT, Biochem. J. *43*, 193, 1948.
9. A. J. P. MARTIN and R. PORTER, Biochem. J. *49*, 215, 1951.
10. R. PORTER, Biochem. J. *53*, 320, 1953.
11. A. TISELIUS, Arkiv f. Kemi 26 B, No. 1, 1948.
12. V. T. RILEY, Science *107*, 573, 1948, *109*, 361, 1949.

13. See for example: L. ZECHMEISTER and M. ROHDEWALD in *Fortschritte der Chemie Organischer Naturstoffe* VIII, 341 (Springer-Verlag, Wien, 1951).
14. S. M. SWINGLE and A. TISELIUS, Biochem. J. *48*, 171, 1951.
15. K. AGNER, Biochem. J. *32*, 1702, 1938.
16. J. B. SUMNER, A. L. DOUNCE, and V. L. FRAMPTON, J. Biol. Chem. *136*, 343, 1940.
17. T. SVEDBERG and J. B. NICHOLS, J. Am. Chem. Soc. *48*, 3081, 1926.
18. T. SVEDBERG and A. TISELIUS, J. Am. Chem. Soc. *48*, 2272, 1926.
19. A. TISELIUS, Nova Acta Soc. Scient. Reg. Upsaliensis Ser. IV, Vol. 7, No. 4, 1930.
20. R. S. ALM, R. J. P. WILLIAMS, and A. TISELIUS, Acta Chem. Scand. *6*, 826, 1952.
21. F. C. BAWDEN and N. W. PIRIE, Proc. Roy. Soc. (London) *B 123*, 274, 1937.
22. C. FROMAGEOT, Biochim. et Biophys. Acta *11*, 95, 1953.

Tryckt den 30 september 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 50

Read 10 March 1954

Enzyme systems conducting the aerobic respiration of roots of wheat and rye

By H. Lundegårdh

With 14 figures in the text

1. Introduction

As shown by Lundegårdh and Burström (1933, 1935) the aerobic respiration of wheat roots consists of two fractions or groups of processes, viz. the *ground respiration* and the *anion respiration*, which are easily separated owing to their quite different responses to cyanide, azide, and carbon monoxide. The anion respiration comprises about 50–75 % of the total respiration. It is completely inhibited by 10^{-5} m cyanide (HCN). This system is responsible for the active uptake of salts against a concentration gradient and for at least 90 % of the growth activity (Lundegårdh 1940, 1949a). The remaining 25–50 % of the total aerobic respiration is not affected by concentrations of HCN up to about 10^{-2} m, a fact clearly demonstrating the existence of a group of respiratorical processes in plants proceeding under complete inhibition of the anion respiration. Already at an early date (1935) it was concluded that the anion respiration is conducted by heavy metal enzymes. The identity of the active system with the cytochrome-cytochrome oxidase system was shown by experiments with inhibitors (e.g. the reversal in light of the CO inhibition; Sutter 1950) and finally by direct spectroscopical measurements (Lundegårdh 1951, 1952, 1953, 1954).

The fact that enzyme systems other than the cytochromes are able to accomplish an end-oxidation of carbohydrates does not, of course, eliminate the possibility of intermediate stages or junctions coordinating the non-cytochrome system (II) with the cytochrome system (I), a possibility which has been discussed in previous communications (Lundegårdh 1952, 1953). We know at present a great deal of system I and its oxidase, but very little about system II, particularily regarding the oxidase II. It was, however, tentatively assumed (Lundegårdh 1953a) that flavoproteins are involved either as intermediary catalysts or/and as oxidase. One of the fundamental questions was here to ascertain whether or not a quantitative relation exists between the concentration of flavoprotein in the objects and the intensity of the ground respiration. Similarily it had to be experimentally shown that the anion respiration is quantitatively related to the concentration of cytochrome. The experimental approach to the problem was facilitated by the fact that the activities of the enzyme systems are subjected to wide fluctuations which are induced by the conditions of growth and development.

It was shown several years ago (LUNDEGÅRDH & STENLID 1944) that wheat roots contain large quantities of *peroxidase*, and I have shown recently (LUNDEGÅRDH 1954) that of the total percentage of protohematin, determined after addition of pyridine, the peroxidase lays hold of at least 50 %, sometimes less but frequently more. The rest of the protohematin derives from the cytochromes *b* and *dh* (probably identical with succindehydrogenase; see LUNDEGÅRDH 1954). The function of peroxidase in the living cells is still very little known but this enzyme has to be discussed in connection with the other hemin enzymes.

2. Technical data

The experimental technique was the same as in previous investigations on the cytochrome system (1951–1953). The absorption spectrum of a thick bundle of roots was recorded by means of a rapid and very sensitive spectrophotometer built for this purpose. The seedlings were grown under strip lamps in a complete nutrient solution and the roots were harvested at a total length of 10 to 12 cm. Roots from 14–15 seedlings were handled as a single braid and cut into lengths of 20 mm up to a height of 60 mm. The moist bundles were inserted into a 16 mm wide quartz tube (length 60 mm). The tigthly packed root bundle, consisting of a few hundred pieces, is held in a firm position in the middle of the quartz tube by means of wads of cotton. The tube is closed by rubber stoppers provided with glass tubes for conducting of a stream of solution through the closed tube.

Before applying the stoppers the roots are evacuated and refilled with water. The removal of air bubbles between the single roots and in the intercellulars facilitates the drainage of the

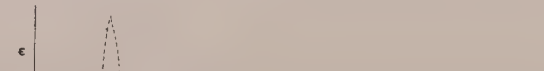


Fig. 1. "Crude spectrum" of a 16 mm thick bundle of wheat roots. ——— Spectrum in aerated 0.025 m. KCl + 0.06 m. KH_2PO_4. At 410–425 mμ the Soret-bands of predominantly oxidized cytochromes *c*, *b*, and *dh*, at 450–455 mμ band of flavoprotein, at 482 mμ band of unknown dye (possibly carotene complex), present in all roots, at 550 and 565 mμ weak reduced bands of cytochromes *c* and *b*. – – – – Spectrum under complete anaerobiosis. Reduced Soret-bands of *b*, *dh*, and *a*, absence of flavoprotein band, reduced joined bands of *c* (visible as hump), *b*, *dh*, and *a*.

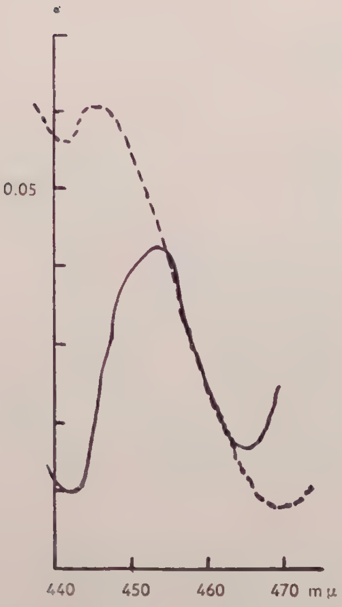


Fig. 2. ——— Oxidized band of flavoprotein in aerated salt solution. ---- Reduced band of cytochrome oxidase instead of the disappeared flavoprotein band at complete anaerobiosis. Wheat roots.

bundle and makes it more transparent. The mode of operation of the automatically recording spectrophotometer is briefly described in a previous paper (1953 a).

"Crude" spectrograms are those directly recorded against a reference beam which has penetrated a sufficiently thick layer (mostly 8–9) of filter paper (see Fig. 1). The crude spectrograms show under aerobic conditions a high joined γ-band of the cytochromes *c*, *b*, *dh*, and *a*. The γ-band of the cytochrome oxidase at 442–444 mμ is slightly interfering with the band of flavoprotein at 455 mμ. Under anaerobical conditions the α-bands of the cytochromes *c*, *b*, and *dh* are clearly recorded in the region 550–571 mμ, but the α-band of *c* is commonly covered by the joined bands of *b* and *dh*. The α-band of *c* may be separately studied on spectrograms recorded immediately after shifting over from aerobiosis to anaerobiosis, because the reduction of the group *a–c* runs much faster than the reduction of the group *b–dh* (cf. LUNDEGÅRDH 1953 c).

Under complete anaerobiosis the flavoprotein band at 455 mμ disappears, and reappears at aeration.

3. Calculation of the apparent molar concentrations of the enzymes

The molar quantity of *flavoprotein* present in the roots was calculated from the difference in extinction at 455 mμ, viz. ε aerobic − ε anaerobic (see Figs. 2 and 3). The molar extinction coefficient was calculated from determinations of the spectrum of oxidized and reduced lactoflavin (Roche). The coefficient of some known yellow enzymes, and riboflavin, varies within the narrow limits 2.42 to 2.60 × 10^4 (WARBURG & CHRISTIAN 1938).

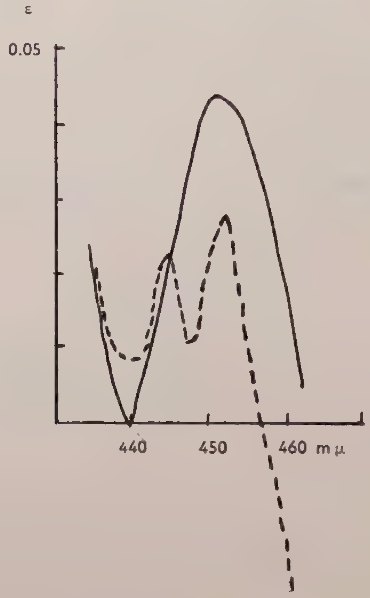


Fig. 3. ——— Oxidized band of flavoprotein in aerated salt solution, no visible band of cytochrome oxidase. ---- At complete anaerobiosis strongly reduced flavoprotein band besides the reduced band of cytochrome oxidase. Roots of corn.

The molar quantities of the prosthetic groups of the *cytochromes* were calculated from the extinction of pure cytochrome *c* at 418 and 550 mμ in the difference spectrum ("ox/red spectrum" or ε red − ε ox). As previously shown (LUNDEGÅRDH 1951–1953) the computation of the ox/red spectrum is the only way of eliminating the background in the spectrum of living organs. Due attention must here be paid to colour changes caused by non-enzymatic substances in the root, e.g. flavanons (cf. LUNDEGÅRDH & STENLID 1944). These substances do not give sharp bands, however. If the height of the bands of the cytochromes are measured in narrow intervals, e.g. the difference 550–540 mμ regarding cytochrome *c*, the contaminations do not disturb the results. The molar extinction coefficients of cytochromes other than *c* are not exactly known, but those of the prosthetic groups are only little varying (cf. LEMBERG & LEGGE 1949). Before accurate biochemical determinations are at hand it seems more appropriate to make the calculations from a medium figure or, for the computation of relative values, to compare the heights of the α-bands.

If the approximate concentration of an enzyme in the living roots is calculated in terms of molar extinction the resulting value is referred to a homogenous solution, but the roots do not fill the recipient to more than 55–65 %. The actual concentration is thus nearly twice as high as the calculated one. The scattering of the light in the living organ creates an inevitable constant error, the magnitude of which is difficult to ascertain. Fortunately all results, also from determinations of the respiration, can be expressed per unit ideal volume, viz. per litre tightly packed roots.

The enzymatic activity of *peroxidase* is ordinarily determined by the purpurogallin method, the accuracy of which, however, has been questioned. No method was hitherto pointed out for determination of the percentage of peroxidase in living organs. Peroxidase normally holds trivalent Fe and for its reduction strong agents, e.g. dithionite, have to be used, which destroy the

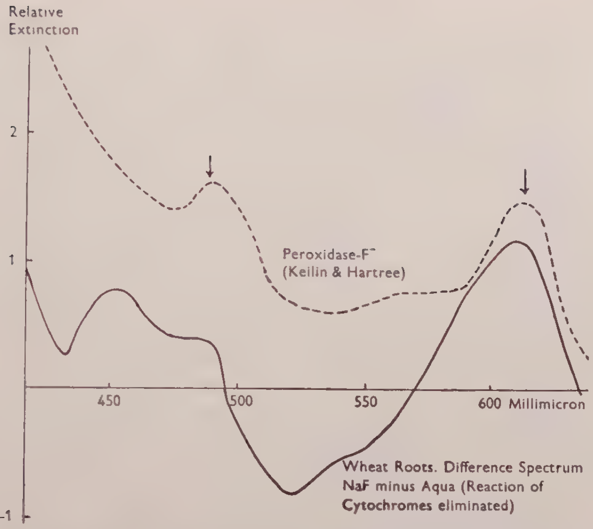


Fig. 4. ——— Fluoride-peroxidase spectrum in a 16 mm thick bundle of wheat roots, computed as difference spectrum between roots treated in 0.05 m. NaF at pH 3.4 and roots in aerated salt solution. – – – – Approximative reproduction of spectrum of prepared peroxidase-F^- according to KEILIN & HARTREE (1951), for comparison.

living structure. According to KEILIN & HARTREE (1951) peroxidase preparations form complex products with CN, N_3, and F. These complexes have characteristic spectra and may be used for quantitative determinations. According to my previous observations (LUNDEGÅRDH 1953a) the fluoride complex is formed in the living roots, if they are soaked in a solution of 0.02–0.05 m. NaF at pH 3.3–4.0 (see Figs. 4 and 5). Measurements are conveniently made on the band 612 mμ, e.g. the difference 612–540, or 612–580, corresponding to the extinction coefficients 0.24×10^4 and 0.175×10^4 respectively. The results were corroborated by the determination of the total protohematin in pyridine plus dithionite (see LUNDEGÅRDH 1954). The percentage of peroxidase is within about 15 % in agreement with what is left after subtraction of the values of the cytochromes *b* and *dh* which together with peroxidase make up the total protohematin.

The spectrophotometric work thus comprises the following steps:

1. Record of the absorption spectrum in aerated 0.01 m. phosphate at pH 5 to 6.
2. The same in O_2-free phosphate.
3. The same in aerated 0.05 m. NaF at pH 3.3–4.0.
4. The same in 15 % pyridine plus dithionite.

As the Speedomax instruments were charged with logarithmically graduated paper difference spectra are calculated by simple subtraction. 2. minus 1. gives the height of the reduced bands of the cytochromes from the level of aerated salt solution (= 70–80 % oxidized; see LUNDEGÅRDH 1953a) to the level of 100 % reduction. 2. − 3. gives the total height of the reduced bands, as fluoride conveys a complete oxidation (see LUNDEGÅRDH 1953a). Because fluoride develops a prominent peroxidase spectrum with a minimum between 500 and 570 mμ (see Fig. 5) the calculation is not quite simple, for which reason the quantity of cytochrome was mostly calculated from 1. − 2. after multiplication with the factor 1.3. 3. − 1. gives the peroxidase and 4. − 1. the total protohematin.

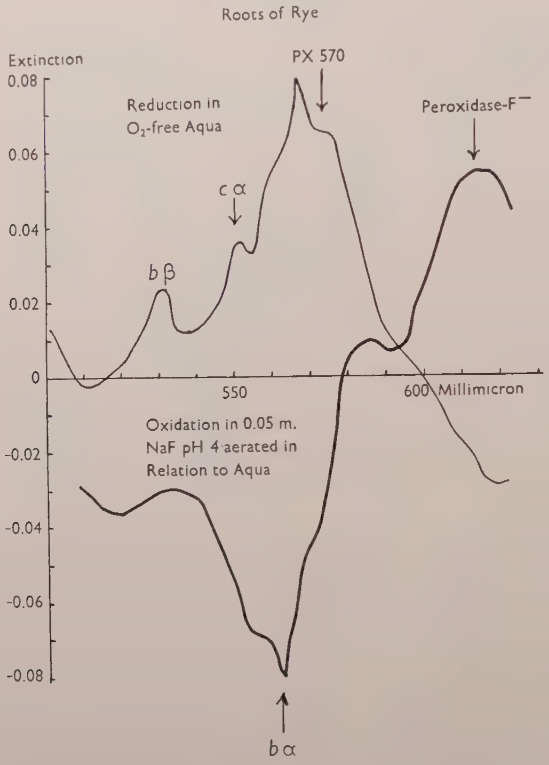


Fig. 5. Difference spectra of a 16 mm thick bundle of roots of rye. Upper spectrogram difference between roots under anaerobiosis and roots in aerated salt solution. α-bands of cytochromes *c*, *b* and *dh*, and *β*-band of *b*. Lower spectrogram difference between roots in NaF and roots in aerated aqua. The cytochromes are strongly oxidized, *c* and *b* more than *dh*; the fluoride-peroxidase band at 612 mμ strongly developed.

4. Determination of the respiration

The ground respiration and the anion respiration were determined in 50 cc. Quickfit flasks. Roots in a quantity of about 1 g fresh weight (50–60 mg dry weight) were enclosed in aerated solutions of 0.01 to 0.05 m. potassium chloride and slowly rotated at 20°C. After ½ or 1 hour the quantity of consumed oxygen was determined by the Winkler method, from which the total respiration per 10 mg dry weight 1 h is calculated. In parallel samples 0.001 m. KCN was added to the solution, resulting in a value for the ground respiration. The anion respiration is calculated as the difference between the total repiration and the ground respiration.

5. The experimental basis of the concept of ground respiration and anion respiration

HILL & HARTREE (1953, p. 133) claim that "no sharp distinction is justifiable" between ground respiration and anion respiration and that the distinction is "highly artificial". They refer to observed "difficulties in interpreting the effect of inhibitions on respiratory processes". This is no conclusive argument, however. When it has been experimentally shown that one fraction of the total aerobic respiration keeps up against a concentration of cyanide at least 1000 times higher than the concentration which is completely inhibiting the other fraction, the conclusion is well founded that the two processes are conducted by different enzyme systems (cf. JAMES 1953). It has been shown (LUNDEGÅRDH 1951–1952, 1953a) that the anion respiration is identical with a cytochrome-cytochrome oxidase system of similar pattern as that working in the heart muscle of animals. The extremely high sensitivity to cyanide is mainly caused by the low value of the dissociation constant of the complex cytochrome oxidase × HCN (HORECKER 1950), whereas cytochrome *dh* and cytochrome *b* very slowly react with cyanide. It was experimentally shown that in intact seedlings of wheat, the roots of which were surrounded by a solution of 0.0001 to 0.001 m. KCl plus 0.001 to 0.002 m. KCN, the anion respiration—and the active salt absorption—are *instantaneously* inhibited. The remaining ground respiration runs at constant speed for at least two hours and is not affected by a considerable rise in the concentration of the cyanide.

In the "normal" anion respiration the complete cytochrome system is at work. As previously shown (LUNDEGÅRDH 1952–1954) it is now possible to disjoint the cytochromes *b* and *dh* from an interaction with the cytochromes *c* and *a*, e.g. with fluoride or dinitrophenol. Roots treated with suitable concentrations of these inhibitors still may show a considerable anion respiration, but they have lost the power of active salt absorption (cf. STENLID 1948–1950). Because fluoride and dinitrophenol uncouple the succinodehydrogenase electrons (and protons) must be delivered from some other dehydrogenase system and it was tentatively assumed that cytochrome *c* and/or the "Slater factor" (SLATER 1949) serves as a junction between the cytochrome system and other pathways of electron transference, e.g. a system consisting of flavoproteins and cozymase. This would give a linkage between the two main systems, ground and anion respiration, too, because flavoprotein is undoubtedly one of the catalysts of the ground respiration (see below).

Other possibilities of an "abnormal" or fragmentary anion respiration is the reduction of cytochrome *c* by circulating substances as ascorbic acid or glutathion. As cytochrome *c* is only little affected by cyanide the possibility cannot, of course, be completely excluded that it participates in the ground respiration too. In that case, however, a cytochrome oxidase which is insensitive to cyanide would be operating in normal roots. Experiments on *the reversal of the cyanide inhibition* of the anion respiration at prolonged exposure (LUNDEGÅRDH 1950b), later completed by yet unpublished series, throw some light upon this question.

If the roots of intact plants are left in a solution of KCl plus 0.0001 m. KCN the inhibition of the anion respiration and of the active absorption of chloride is slowly reversed (see Fig. 6). The reversal is accelerated in higher concentrations of oxygen and retarded at low concentrations. The reversal is not due to a disappearance of cyanide from the aerated solution or in the ascending sap stream. This is shown by experiments in which new quantities of cyanide were added in order to maintain a constant concentration of CN. The same quantity of cyanide which conveys a complete inhibition of the anion respiration in the beginning of the experiment now develops only a strongly reduced inhibition (see Fig. 6). Similar results were obtained with azide, in which the recovery, however, proceeds at a slower rate.

The phenomenon of reversal of the cyanide inhibition was tentatively explained as an intracellular adaptation of the cytochrome system (LUNDEGÅRDH 1950b). It is

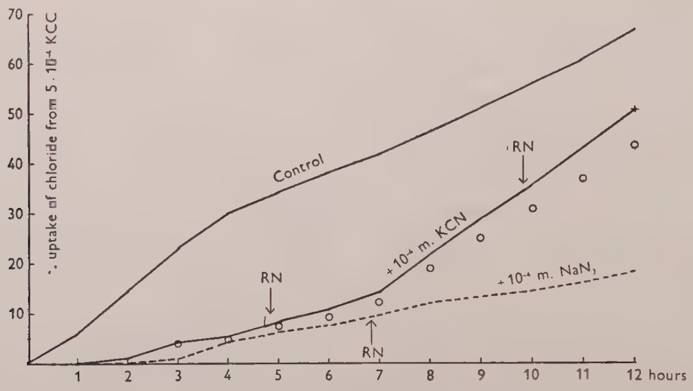


Fig. 6. Diagram of a series of experiments in circulation vessels with intact seedlings of wheat. The vessels contain 100 cc. $5 \cdot 10^{-4}$ m. KCl as standard. The uptake of chloride from the 14 plants in each vessel was continuously recorded by means of electric conductivity (see LUNDEGÅRDH 1949c) and electrometrical titration. The values on the ordinate show how many per cent of the original solution have been absorbed by the plants. To some vessels KCN or NaN_3 was added up to a total concentration of 10^{-4} m. In these vessels the solution of KCl plus inhibitor were renewed at the points indicated by RN and an arrow. The circles accompanying the KCN-curve refer to the electrometric titrations, the curve itself to the conductivity records. The results illustrate the reversal of the inhibition of the anion respiration.

known that the affinity of the hemin to cyanide varies tremendously owing to the mode of linkage to the protein, cytochrome oxidase being extremely sensitive and cytochrome *c* being almost insensitive under physiological conditions, with the cytochromes *b* and *dh* somewhere in between. The phenomenon of reversal points to a rearrangement of the molecular configuration of the cytochrome oxidase, and possibly also of *b* and *dh*, to a more *c*-like structure.

The physiological observations were now completed by spectrophotometric records. Bundles of roots were held in constantly flowing solutions of 10^{-5} m. HCN and the difference spectrum (see above) was recorded in increasing intervals. The spectrophotometric records definitely prove a reversal of the reduction of the cytochrome system. In one experiment a 30 % oxidation of cytochrome *c* was recorded after 10 hours' treatment with 10^{-5} m. HCN. In another experiment cytochrome c was 25 % oxidized after only 3 hours (Fig. 7). The recovery of the cytochromes *b* and *dh* runs slower than that of *a* and *c*. The fact that the active absorption of salts is recovered synchronously with the increased oxidation of cytochrome *c* proves, however, that also the activities of the cytochromes *b* and *dh* are intact.

Our investigations as to the reversal of the inhibition of the cytochrome system open new aspects on the adaptation processes going on in the protoplasmatic metabolism and on the existence of different types of one and the same enzyme ("families of enzymes"), but the results cannot be alleged in support of the belief of HILL & HARTREE (1953) that there is no distinction between ground respiration and anion respiration. For, it has been experimentally shown (see above) that cyanide inhibits instantaneously, not gradually, and that the ground respiration remains unaffected from the beginning. The adaptive appearance of a cytochrome system insensitive to cyanide is a slow working process, extended over a period of several hours. It has also been experimentally shown that the partially recovered anion

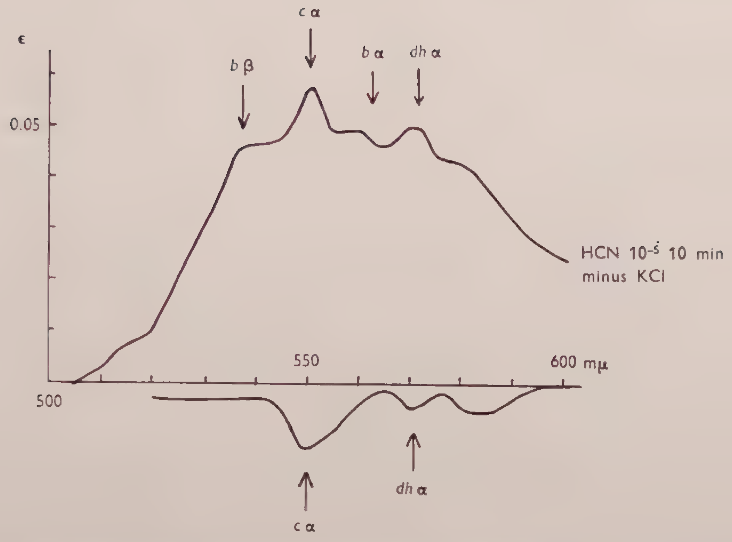


Fig. 7. Reversal of the inhibition of the cytochrome system in aerated 10^{-5} m. HCN (neutralized KCN) continuously flowing through the recipient with the root bundle. Upper curve shows the reduced joined bands of the cytochromes 10 minutes after the addition of cyanide, lower curve shows the difference between the spectrum after $2\,^3/_4$ hours treatment and the spectrum at the start. The reduction of cytochrome *c* is 25 % lowered.

respiration can be again completely inhibited by strong solutions of HCN. The sensitivity to cyanide is consequently not lost altogether, as it is from the beginning with regard to the ground respiration, but only considerably lowered.

The discussion as to a competition between oxygen and an inhibitor goes back to the idea originally held by WARBURG (1934) that the respiratory enzyme (= cytochrome oxidase) besides of its oxidizing qualities behaves like hemoglobin, viz. binds molecular oxygen at its surface, the oxygen then being reduced by the ferrous hemin group. WARBURG believes, as do a number of later investigators (see LEMBERG & LEGGE 1949, KEILIN 1953), that only the reduced oxidase binds molecular oxygen. Under the assumption that carbon monoxide combines with ferrous hemin, O_2 and CO would then compete at the surface of the enzyme. If now the common assumption is adequate that cyanide combines with the ferric hemin, this would according to WARBURG and later defenders of the oxygenating qualities of the cytochrome oxidase exclude a competition between oxygen and cyanide.

Experiments performed twenty years ago in this laboratory now clearly show that the intensity of the cyanide inhibition of the anion respiration is regulated by the concentration of oxygen in the medium (LUNDEGÅRDH & BURSTRÖM 1935, LUNDEGÅRDH 1945, p. 39). The anion respiration is completely inhibited by 1.5×10^{-5} m. KCN at an O_2-concentration of 0.250 millimols × lit.$^{-1}$ (= normal saturation in contact with air) but the inhibition is appreciably reversed at an O_2-concentration of 1.0 millimols × lit.$^{-1}$ (see Fig. 8). As mentioned above the same phenomenon was observed in time records of the recovering anion respiration (LUNDEGÅRDH 1950b): the recovery proceeds considerably more rapidly if the oxygen concentration is high.

The oxygenation of hemoglobin is chiefly due to the protein globin, not to the hemin itself (LEMBERG & LEGGE 1949, p. 207). Similarly it may be assumed that the

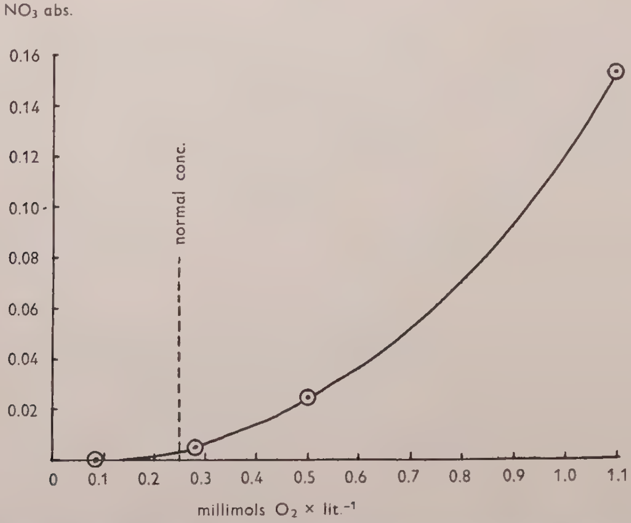


Fig. 8. The competitive effect of the O_2-concentration on the inhibition of the anion respiration by cyanide. From experiments by LUNDEGÅRDH & BURSTRÖM (1935, table IV). The basic solution was 0.00125 m. KNO_3 plus 0.00015 m. KCN, only the oxygen concentration was varied. It has to be emphasized that even at *c.* 0.1 millimol O_2 × lit.$^{-1}$ the anion respiration and the active uptake of anions proceed at normal speed in the absence of KCN.

protein of the cytochrome oxidase binds molecular oxygen on a point at the surface adjacent to the hemin. The competition between oxygen and cyanide is explained if we assume that also molecules of HCN are attracted to this point. The attraction of molecules of oxygen and inhibitors to the cytochrome oxidase is certainly of a much lower intensity than is the oxygenation of hemoglobin but will nevertheless contribute to the accessibility of the oxygen. Observations of the time course of reoxidation from complete anaerobiosis and of cytochrome reduction with cyanide teach us that these processes are running extremely fast (see LUNDEGÅRDH 1952, 1953a), a fact pointing to the cooperation of factors speeding up the diffusion. Even a comparatively modest oxygenation of hemin enzymes will undoubtedly provide for such an accelerated diffusion. It may be mentioned incidentally that the roots do not contain measurable amounts of hemoglobin, as compared with the nodules of legumes or certain yeasts and fungi (KEILIN 1953).

A molecular attraction between the cytochrome oxidase and oxygen does not in itself interfere with the electron transference, only raise the molar activity of the oxygen above the level of the medium. The effect will be comparable with the promoting effect of an colloidal adsorbens on the availability of salt ions to the roots. The comparatively weak attraction of oxygen is illustrated by the fact that a concentration of 10^{-5} m. HCN completely inhibits the anion respiration at a concentration of 2.5×10^{-4} m. of oxygen or 25 times more. The competitional effect is noticed if the oxygen concentration is raised to 50–100 times the concentration of cyanide.

The competitive effect of O_2 contra HCN observed on "normal" cytochrome oxidase may be characterized as a partial shielding off of cyanide from the prosthetic group. What we have observed as a reversal of the cyanide inhibition of the anion respiration is simply a reinforcement of this shielding effect. Because the reversal is

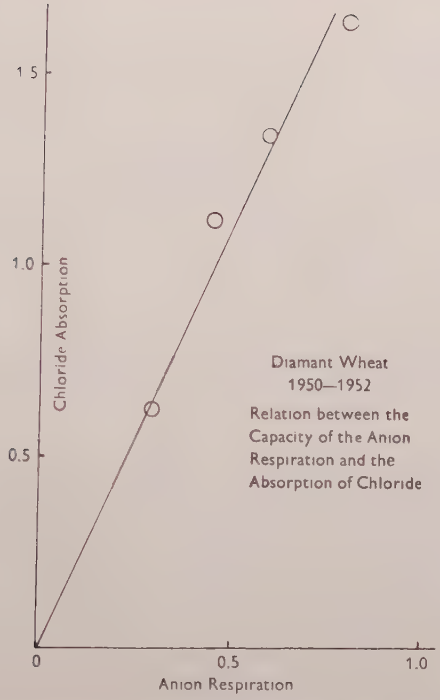


Fig. 9. Diagram from experiments on chloride absorption and anion respiration performed in different seasons and at varying content of cytochromes.

not accompanied by spectral changes the configuration of the prosthetic group is most probably not altered, only the configuration of the point on the apoenzyme, at which the oxygen molecule is attracted. The slow proceeding of the rearrangement or adjustment of the cytochrome oxidase points to a cooperation of metabolic processes and speaks against the coexistence of two types of cytochrome oxidase in non-inhibited roots. It is unlikely that the oxidase II, which is conducting the ground respiration, is a pre-existing cytochrome oxidase resistant to medium concentrations of cyanide, because it would then immediately take over the responsibilities of the "normal" cytochrome oxidase (see above). But the possibility cannot be excluded that some other autoxidizable cytochrome, resistant to cyanide, is directly linked to the flavoprotein system conducting the ground respiration. Attention is here called to the fact that the cytochromes *b* and *dh* are fairly resistant to cyanide and that *b* may be directly oxidized by oxygen.

All attempts to identify the oxidase II with a copper enzyme (polyphenol or ascorbic acid oxidase) have hitherto been unsuccessful, too. Already the pronounced insensitivity of the ground respiration to CN and CO does not support the idea of a copper enzyme. According to HILL & HARTREE (1953, p. 141) plant tissues generally show either peroxidase activity or polyphenol oxidase activity. The high percentage of peroxidase in roots would then make the presence of p.p.o. less probable.

Recent investigations on the respiration of the spadix of *Arum maculatum* (JAMES & BEEVERS 1950, HACKETT & SIMON 1954) have revealed the presence of an aerobic oxydation depending at least in large part upon a flavoprotein enzyme. Cytochrome

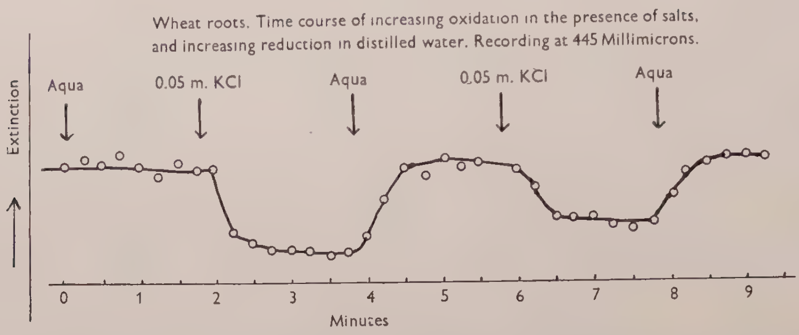


Fig. 10. Diagram showing the rapid response of the oxidation-reduction level of cytochrome oxidase in living roots at a change in the salt content of the solution.

is believed to be absent. Flavoproteins are known to be autoxidizable but it was earlier assumed that the turnover rate of flavoprotein enzymes is too slow for an effective aerobic respiration. It would seem that the recent results indicate the existence of flavoproteins of considerable oxidative activity (cf. HORECKER 1950).

Roots of cereals—as most other plant organs—contain flavoproteins appearing as bands at 380 and 455 mμ in the crude spectrum (Figs. 1–3). The band at 455 mμ disappears more or less completely at anaerobiosis, probably because of the formation of a leucosubstance. The flavoprotein in roots thus behave like an enzyme operating as a carrier of electrons and protons between a dehydrogenase system and oxygen, possibly via a separate oxidase II (see above). The nature of the dehydrogenase is not known but it may partly be succinic dehydrogenase (or cytochrome *dh*), because this enzyme is rather resistant to cyanide. It was tentatively assumed that the flavoprotein is linked to cozymase (Co I) as a hydrogen donor (cf. WAYGOOD 1950, SLATER 1949, 1950) and thus operates from other starting points in the intermediate tricarboxylic cycle and/or the chain of glycolysis than the cytochrome system. Observations on the continued activity of *a* and *c* at uncoupled *b* and *dh* (see above) point to an activity of flavoprotein as a cytochrome *c* reductase, too.

6. The anion respiration and the active absorption of anions

The relations between the activity of the cytochrome system and the active absorption of anions have been extensively demonstrated in previous papers. It was shown that these relations include two phases, 1. the coenzymatic effect of anions, and 2. the active (polar) transport of salts. These effects are not necessarily coordinated, but phase 2 is based on phase 1 which is assumed to be universal. In suitably prepared organs (see earlier work by LUNDEGÅRDH and by ROBERTSON) the anion respiration shows an approximately linear relation to the salt absorption, if measurements are made in the course of one or two hours (Fig. 9). The start period of the active anion absorption, amounting to 10–20 minutes (LUNDEGÅRDH 1949b, 1950b, 1952, 1953a), is reflected in the time course of the displacement of the oxidation-reduction balance of cytochrome *b* (and *dh*). The cytochrome oxidase (and cytochrome *c*) reacts more rapidly, the level of oxidation-reduction of this enzyme is in a few seconds displaced in direction of a higher oxidation, if aqua dest. is exchanged for a solution of 0.05 m. KCl (Fig. 10). This rapid response of the cytochrome oxidase may be reversely repeated *ad libitum*, and supports the conclusion of this enzyme being located in the surface of the cells. The same conclusion is arrived at by experiments with oxidation by living roots of reduced cytochrome added to the medium (LUNDEGÅRDH 1952b, 1953a, p. 132).

The observations of the interaction between salts and added cytochrome *c* on the one hand and the cytochrome system of the roots on the other thus conveys the conclusion that the cytochrome oxidase is located in or very near to the surface membrane of the protoplasm, whereas the cytochromes *b* and *dh* may as well be located in the bulk of the protoplasm or in the membrane of the tonoplast. It is commonly assumed that the total cytochrome system (*a*, *c*, *b*, *dh*) is organized as a structural unit—with the possible exception of *c* as a more ambulatory enzyme—but most investigators are inclined to locate these units in the mitochondria, an hypothesis not well rhyming with the observations of the superficial location of the cytochrome oxidase and of the polar movement of salts from the medium into the vacuole. Both these facts are more compatible with the idea of the cytochrome system fixed in the surface membrane of the cytoplasm (LUNDEGÅRDH 1953a). The minute particles fractionated from homogenized tissues are commonly assumed to be identical with mitochondria or microsomes of living cells but the possibility is still lurking that at least some of the isolated particles are artefacts, composed by substances and enzymes going into segregation when the body of the cell is atomized. If we assume that the cytochrome system or some part of it, e.g. the cytochrome oxidase, has the tendency to move into a boundary between two phases of different hydration, e.g. between phosphatides and aqueous solutions, it will readily appear in isolated "mitochondria". The same property will in the living cell direct the cytochrome system—as a submicroscopical unit of the protoplasm—into any surface, viz. the outer and inner plasma membrane and the membrane of mitochondria, and it will then depend upon the prevailing metabolic and developmental conditions in which place it will be preferably located. I have advanced the suggestion that just the location of the cytochrome system in the cell decides its general metabolic fate.

The location in the surface membrane of mitochondria or microsomes must tremendously promote the intensity of oxidation and this is probably one of the best reasons supporting the assumption of the cytochrome system of the heart muscle of animals being located in the mitochondria. Such cells are obviously not endowed with the power of polar salt accumulation and the interaction between the salts and the cytochrome system is restricted to phase 1 (see above), viz. the common coenzymatic effect of salt anions. A location of the cytochrome system preferably in a membrane enclosing the protoplasm or surrounding the vacuole would be very convenient for salt accumulation because true accumulation cannot be maintained without an effective diffusion barrier impeding the back-flow of once accumulated salt. Plant cells do not need the high total respiratory activity of many animal cells but they need a mechanism for active salt absorption. A localization of the cytochrome system in a plasma membrane gives a lower total respiration but in return for that creates the power of accumulating salts.

The non-metabolical exchange capacity in respect of salts probably comprises the bulk of the protoplasm (see above), but this fact cannot be alleged as a conclusive argument against a localization of the cytochrome system in the outer protoplasma membrane. It can be calculated from the concentration of the cytochromes in the roots that a localization of the cytochrome system in the cell surface would leave ample room for other systems and substances which are participating in the non-metabolic absorption and exchange of ions. The points in the membrane where the cytochrome "units" are located would be at a distance from one another corresponding to a concentration of only about 10^{-4} m. but they would successfully contribute to an enhanced import of salts into the protoplasm. The limited concentration of points in the membrane which serve as entries of non-metabolically adsorbed ions—corresponding to a surface concentration of about 10^{-3} m. as calculated from the surface potential (LUNDEGÅRDH 1938, 1940, 1941)—creates an effective barrier against back-flow of the actively absorbed salts. Since the cortex cells of the roots, and cells of storage organs, too, have a very thin layer of protoplasm between the outer and inner membranes, the possibility cannot be rejected that half of the cytochrome system is located in the outer membrane (*a* and *c*) and the other half (*b* and *dh*) in the vacuole membrane, but few arguments are supporting such a hypothesis.

7. Quantitative relations between cytochromes *b* and *dh* and the anion respiration

The intensity of the anion respiration is directly proportional to the volume concentration of cytochrome *b* in the roots (Fig. 11). In terms of molar concentration the relations between the consumption of oxygen per 1 cc. and 1 minute and the cytochrome *b* is as follows:

$$\frac{\text{mol } O_2}{\text{mol cyt. } b} = 170.$$

Four electrons are required for the reduction of 1 mol O_2 to 2 mol H_2O. This gives a turnover number per minute of $4 \times 170 = 680$, calculated per 20° C (cf. LUNDEGÅRDH 1953a, p. 137).

The spectroscopic behaviour of the wheat roots has been closely followed during a period of more than three years. Variations have been observed not only in the total amount of cytochrome per unit fresh weight but also in the quantitative relations of the single cytochromes. In the complete system, consisting of *a*, *c*, *b*, and *dh* (+ succinic dehydrogenase), two groups can be distinguished, *a–c*, and *b–dh*. The former group is less varying than the latter one. The quantitative relations between the two components of each group, which have been more closely studied regarding *b–dh*, are comparatively constant. The variations in the anion respiration, and concomitantly in the active salt absorption, are closely reflected in the varying volume concentrations of the group *b–dh*.

As to external and internal causes of variations the following may be said.

The quality of the seeds is exceedingly important. A number of still incompletely known circumstances are here interfering. One important factor is the climate during the vegetation period. Another factor is the climate, particularly the humidity, in the season of harvest. The high activity, both in regard of cytochrome concentration and active salt absorption, of seeds yielded in 1949–51 could be correlated with the favourable seasons in these years. The cool summer 1952 in combination with too much rain in the autumn yielded seeds of less good quality. These seeds showed a lower percentage of germination and the roots did not grow as excellently as in previous years. Frequent determinations of the concentration of the cytochromes and of the

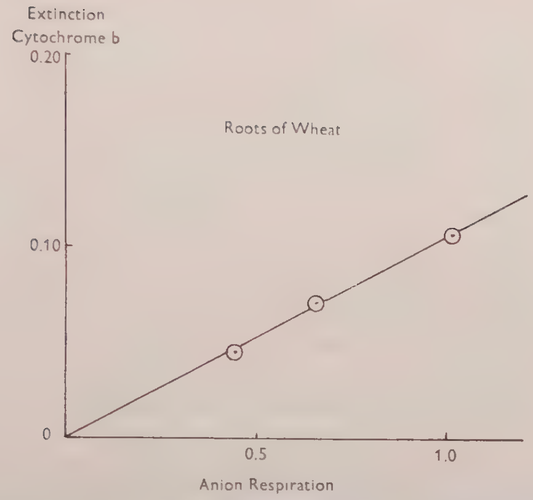


Fig. 11. The covariation of cytochrome *b* and the anion respiration under different conditions of development.

anion respiration and active absorption of chloride showed an activity of only about 50 % of that obtained in 1950–51. The total quantity of hemin—predominantly protohematin (LUNDEGÅRDH 1954)—is considerably more constant than the percentage of cytochrome. According to the conditions of growth and development more or less of the total hemin is turned over into cytochrome. What remains of hemin appears as peroxidase, with a possible reserve of unspecified protohemin IX. This reserve amounts to maximum 15 % or so and may be practically absent in some cases. The percentage of cytochrome, in particular of *b* and *dh* (see above), gives a measure of the physiological activity of the seedlings, because the anion respiration is apparently a condition for what may be called the "power of creative work".

The percentage of peroxidase is always high in the wheat roots and amounted in the years 1949–51 to about the same value as the sum of the cytochromes *b* and *dh*. In the following years, at sinking percentage of cytochrome, the peroxidase is predominating, amounting to about 200 % of $b+dh$. Because of the comparatively constant total quantity of hemin the cytochromes $b+dh$ and the peroxidase are varying inversely. The possible biochemical consequences of this will be discussed in Section 9.

The quantities of total hemin turned over into cytochromes are not only exposed to after-effects of the climatic conditions, under which the mother-plant was developed, but also to the nutritional state of the seedling itself. A series of experiments were performed with increasing concentrations of mineral nutrients (Fig. 12). The percentage of cytochrome *b* is low if the roots are developed in distilled water, but rises about 100 % after the addition of nutrient salts (complete solution) to a concentration of 1.8 g per litre which corresponds to that of a normal nutrient solution promoting optimal growth. At higher concentration of mineral nutrients the percentage of cytochrome is again diminished. These experiments were performed with seedlings of rye, in the roots of which cytochrome *b* amounts to about 23 % of the total hemin and cytochromes $b+dh$ amount to the same quantity as the peroxidase.

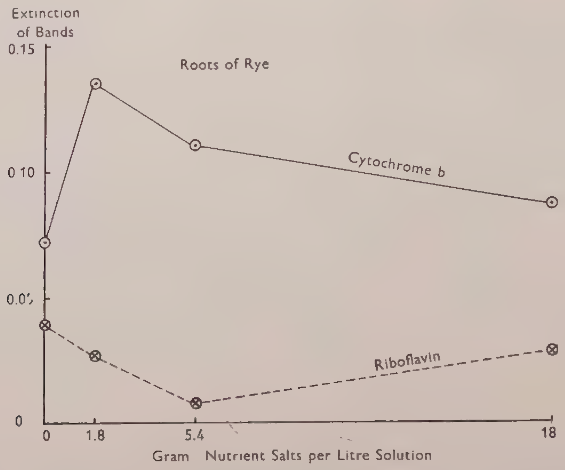


Fig. 12. The relation between the concentration of the nutrient solution and the volume concentration of cytochrome *b* and riboflavin in roots of rye.

8. Quantitative relations between flavoprotein and the ground respiration

In Fig. 12 are also plotted figures of the relative concentration of flavoprotein and of the intensity of the ground respiration (total respiration minus anion respiration). The anion respiration varies synchronously with the height of the reduced bands of cytochrome *b* and the ground respiration varies synchronously with the height of the flavoprotein band at 455 mμ. Table 1 gives similar figures from a series with wheat roots.

The turnover number per minute of $\frac{\text{flavoprotein}}{\text{ground respiration}}$ amounts to about 90 in the case of wheat and about 70 in the case of rye. It is about 50 % lower than the turnover number of cytochrome *b* (see above) and amounts only to about 12 % of the turnover number of the cytochrome oxidase, figures compatible with the considerably lower effectivity of the ground respiration system and a certain sluggishness of

Table 1. Co-variation of flavoprotein and ground respiration. Experiments with wheat roots, exposed to varying growth conditions (varying concentrations of mineral nutrients).

Extinction at 455 mμ	Ground respiration at 20°C μmol O_2 per 10 mg d.w. and 1 h
0.036	0.54
0.047	0.66
0.083	1.22
0.099	1.49

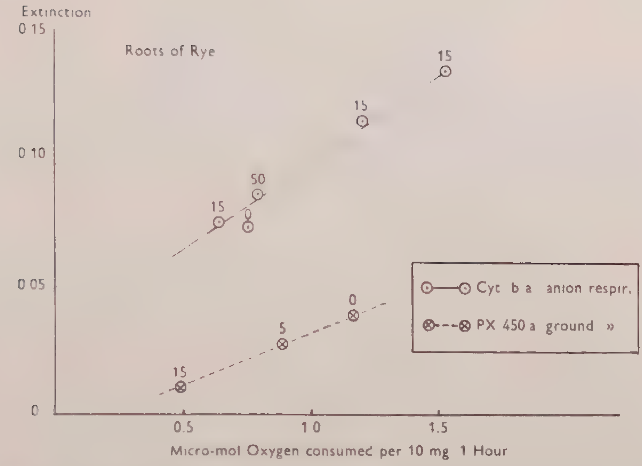


Fig. 13. Quantitative relations between cytochrome *b* and the anion respiration, and between flavoprotein and the ground respiration. The figures refer to the relative concentration of the nutrient solution, in which the roots were grown (cf. Fig. 12). 0 is distilled water and 5 is normal concentration, corresponding to 1.8 g total salt per litre.

the flavoprotein enzymes. The quantities of flavoprotein present in the roots are higher than those of the single cytochromes and may be compared with the total quantity of cytochrome, viz. about 2–4 μmol per kg fresh weight.

A fact of considerable interest is the inverse variation of anion and ground respirations (see Figs. 12, 14). As a result, the total aerobic respiration is comparatively constant. The most striking thing is that the balance between the two systems is effected by means of the formation of more or less of the conducting enzymes, not by retailing more or less of glucose into each one of two preformed enzymatic mechanisms. The results point to the existence of competitional processes in the chains of reactions leading to the synthesis of cytochrome and of flavoprotein.

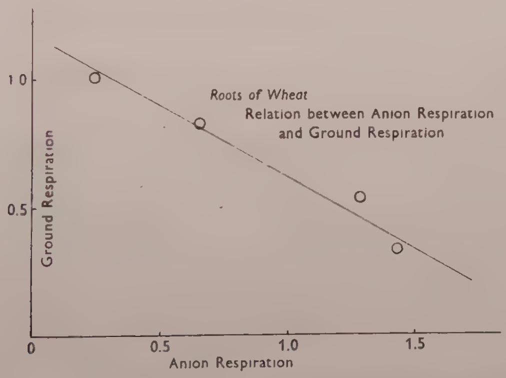


Fig. 14. Diagram showing the inverse relation between anion respiration and ground respiration in a series of experiments with varying growth conditions.

9. Discussion and conclusions

a. The possible role of peroxidase

Peroxidase is invariably present in plant organs, frequently in higher concentrations than cytochromes, but very little is still known about its metabolic task. According to KENTEN & MANN (1949, 1950) peroxidase may act as an oxidizing agent for plant metabolites in cooperation with manganese:

$$\text{(1)} \qquad AH_2 \text{ (peroxidase substrate) } \xrightarrow[+H_2O_2]{\text{peroxidase}} A$$

$$\text{(2)} \qquad A + Mn^{++} \longrightarrow Mn^{+++} \text{ (or } Mn^{++++}) + AH_2$$

$$\text{(3)} \qquad Mn^{++} + O_2 \xleftarrow{H_2O_2} Mn^{+++} \xrightarrow{\text{metabolites}} Mn^{++} + \text{oxidized metabolites.}$$

This scheme may provide for an explanation of the activating effect of Mn^{++} on the *ground respiration* of wheat roots which I observed at an early date (1939). After turning over of roots cultivated without manganese in a solution of 5×10^{-5} m. $MnCl_2$ the ground respiration was increased up to 150–500 %. Hydrogen peroxide may be fed to the peroxidase system from a number of intracellular sources, e.g. flavoprotein enzymes (cf. GALSTON & BAKER 1951). Also ascorbic acid has been included as H donor for the peroxidatic reaction (CHANCE 1948). According to KENTEN & MANN (1953) the system H_2O_2-Mn-peroxidase, however, requires higher concentrations of manganese than the biologically normal ones and may possibly be confined to toxical cases only.

A draw-back to the occurrence of peroxidase respiration is the sensitivity to cyanide. It is claimed that peroxidase is strongly inhibited by 10^{-5} to 10^{-6} m. cyanide or concentrations which are without any effect on the ground respiration. We arrive at the conclusion that common peroxidase is probably not conducting the ground respiration unless a modification of it exists in the living organs which is considerably more shielded off from CN than are peroxidase preparations. In the light of what has been said above on "families of enzymes" such a possibility has to be considered. A pronounced insensitivity of "living" peroxidase to cyanide is, however, no common property, as shown by experimental observations of the respiration of horse radish.

Horse radish is provided with about two or three times more peroxidase than the roots of cereals but no cytochrome can be detected. There is no trace of reduced bands of cytochrome under complete anaerobiosis or in cyanide. In spite of that slices of horse radish suspended in salt solutions show a considerable cyanide sensitive consumption of oxygen, amounting to about 50 % of the total respiration without cyanide. Even in horse radish, however, a ground respiration, similar to that observed in the roots, is going on (Table 2). The fact that the cyanide sensitive respiration in horse radish is inhibited also by azide, an inhibitor against which peroxidase is claimed to be more insensitive (see the survey in HILL & HARTREE 1953), puts the activity of this enzyme on a basis from which for the present no further conclusions can be drawn.

The question as to a possible cooperation of peroxidase and cytochromes must hence be left open. The fact that the percentage of peroxidase in the roots varies inversely to the intensity of the anion respiration does not speak in favour of a coopera-

Table 2. The respiration of the root tissue of horse radish, as compared with the respiration of wheat roots.

A. Horse radish cut in slices 12 mm in diameter and 1–2 mm thick.

	μmol O_2 per 1 g fresh weight 1 hour
Total respiration (TR)	3.70
Fraction of the respiration inhibited by cyanide and azide (PR)	1.55
Fraction resistent to inhibitors (GR)	2.15

Percentual values.

TR	GR	PR
100 %	58 %	42 %

B. Roots of wheat. Average of 10 experiments with seeds from 1952.

	μmol O_2 per 1 g fr. w. 1 hour
Total respiration (TR)	13.65
Anion respiration (AR)	7.42
Ground respiration (GR)	6.23

Percentual values.

TR	GR	AR
100 %	45 %	55 %

tion between the cytochromes *b* and *dh* and peroxidase. The cytochromes *a* and *c* are far less varying than the last-named group and they are obviously also able to join other chains of dehydration, e.g. via flavoprotein.

Far-reaching conclusions as to the metabolic purpose of enzymes, drawn from their behaviour in vitro, ought to be avoided. The large quantities of hemin stored in peroxidase may be provided with some activity in the living cell of which yet nothing is known. Only combined physiological and biochemical experiments will yield further progress. One possibility is a relation between the peroxidase in the living cell and the attraction and movement of oxygen molecules. Peroxidase has certainly no oxygenating qualities comparable to hemoglobin, however. This possibility was tested by some experiments with horse radish.

A comparison was made between the respiration of solid blocks of root tissue of horse radish and the same volume of tissue cut in 2 mm thick slices (diameter 12 mm). The problem was put as follows: If an oxygenating system exists in the tissue it may be expected that the increase in respiration after slicing of the tissue will be less than the increase in free surface; if not, the increase in respiration will be directly proportional to the multiplied surface. The experiment affirmed the latter thesis, viz. the respiration rose approximately linear to the surface even at low concentrations of oxygen and in quick experiments (10 minutes). The presence of peroxidase in the horse radish does not accelerate the transport of oxygen. The aeration of the tissue is indeed rather poor, but may possibly be better in aerated soil than in full contact with a solution. If the peroxidase molecule attracts oxygen molecules the attraction surely remains at a low level.

Our main conclusion regarding the role of the peroxidase in the metabolism of the wheat roots is consequently that it does probably not "normally" participate in the ground respiration. Experiments with horse radish show (see Table 2) that peroxidase very probably is conducting one fraction of the aerobic respiration which may be inhibited by cyanide, and azide. It must consequently be concluded that a peroxidase respiration may be included in that fraction of the respiration of roots of cereals which is inhibited by cyanide (and azide), viz. the anion respiration. The following calculations will elucidate the possible magnitude of such a peroxidase respiration.

From determinations of the total protohematin in the root of horse radish it can be calculated that the fresh tissue contains about 10 μmol peroxidase per litre. One litre tissue shows a PR (see Table 2) of about 1600 μmol O_2 per 1 hour. From these figures the turnover number of the peroxidase may be calculated, viz.

$$\frac{1600}{10 \times 60} = 2.7 \text{ per minute.}$$

If we wish to compare these figures with the turnover number of flavoprotein and cytochrome in the roots we must introduce a correction for the surface factor. The roots have a diameter of 0.5 mm and the slices of horse radish were 1–2 mm thick. Because the experimentally stated linear relation between the extension of the surface and the peroxidase respiration is probably somewhat decreasing at further dividing of the slices, we multiply the above figure of the turnover number with 2, thus $2 \times 2.7 = 5.4$ per minute. The figures for flavoprotein (ground respiration) and cytochrome *b* (anion respiration) were about 80 and 170 respectively. These systems are thus far more effective than the hypothetical peroxidase system.

Provided that the peroxidase in the roots participates in a similar aerobic respiration as in horse radish we can now calculate its approximate magnitude. Wheat roots on an average contain about 50 % less peroxidase than horse radish, or 5 μmol per litre fresh tissue, corresponding to a calculated respiration of $\frac{2 \times 1.55}{2} = 1.55$ μmol O_2 per 1 g fresh weight 1 hour (cf. the values in Table 2), thus maximally about 20 % of the cyanide sensitive fraction of the respiration. The calculation is fairly approximate but it gives an idea what may be expected from a possible participation of peroxidase in the aerobic respiration.

The biochemists emphasize a pronounced insensitivity of peroxidase to carbon monoxide. This gives an opportunity to test the question as to the participation of peroxidase in the anion respiration. From the experiments performed by SUTTER (1950) it can now be calculated that the values of Q an/O_2 (see LUNDEGÅRDH 1949 b) remain about the same if the anion respiration is inhibited by KCN as if it is inhibited by carbon monoxide. If peroxidase had added something to the cyanide sensitive respiration this would give a lower value of Q an/O_2 than with carbon monoxide. We consequently arrive at the conclusion that a separate peroxidase respiration, if it at all exists, proceeds at such a low intensity that it does not significantly alter the quantitative relations between the cyanide sensitive respiration and the active transport of anions.

b. The quantitative relations between anion respiration and active transport of anions

As peroxidase attracts one anion per atom Fe (SUMNER & MYRBÄCK 1951, p. 422), a quality perhaps characterizing all hematin compounds (LEMBERG & LEGGE 1949, p. 201), the question arises if it may be able to participate in the transport mechanism of salts. Let us first consider what is known about the cytochrome system as a transport mechanism of anions.

As shown by ROBERTSON (1948, see 1951) it may be expected that maximum 4 anions will be transported for each molecule oxygen reduced to water. This value was approximately attained in experiments with slices of tissues but not in roots, in which Q an/O_2 values of 1–2 were not surpassed. I explained these results from an interaction of "native" anions, primarily of organic acids. If native anions are competing with anions coming from the medium, a Q an/O_2 calculated only from the imported anions will give lower values than the theoretical 4 anions. The validity of this explanation was supported by experiments with "distilled water respiration", viz. the intensity of the cyanide sensitive respiration which is claimed to transport only anions of organic acids and inorganic anions inside of the epidermis. It was shown that the intensity of this "anion respiration without absorption of anions from the medium" decreases during prolonged washing of the roots in distilled water, glucose or at low pH (see Table 3). Other experiments showed a marked decrease of

Table 3. Experiments with wheat roots held in distilled water, or glucose, or diluted HCl and then analysed as to the respiration. From LUNDEGÅRDH (1949 b), tables 3 and 4. Averages were taken from the zones 0–30 and 30–60 mm.

Treatment	Respiration (µmol O_2 per 10 mg d.w. 1 h)			AR in % of maximum
	TR (aqua)	GR (HCN)	AR (TR–GR)	
1 day in aqua dest.	2.73	0.96	1.77	100
2 days » » »	2.17	0.86	1.31	74
4 » » » »	1.87	0.87	1.00	57
4 » aqua + 1 day glucose . .	1.58	0.86	0.72	41
2 » in 5 × 10^{-4} HCl	1.57	1.00	0.50	28

Table 4. The level of malic acid in the root tissue at different treatments. The roots were held about four hours in the different solutions. Electrometric titration of sap extracted by boiling water.

Aqua dest.	5×10^{-4} n · H_2SO_4	5×10^{-4} HCl	10^{-2} $KHCO_3$
pH 6.3 0.156 m.	pH 4.8 0.094 m.	pH 4.0 0.075 m.	pH 9.5 0.236 m.

the content of inorganic salts at a prolonged stay in distilled water (LUNDEGÅRDH 1945, 1950a). These were removed by synthetical processes, e.g. NO_3 by proteinization or through the bleeding sap (LUNDEGÅRDH 1943, 1945, 1950a). Organic acids, of which malic acid is predominating, are rapidly disappearing if the roots are held at lower pH-values (Table 4) and reappear at high pH.

The results of Tables 2–4 were calculated on roots of the same length as in the spectrophotometric measurements (about 60 mm).

The experiments in Tables 3 and 4 teach us that the anion respiration is to a large extent choked by the deficiency of mobile ions of salts or organic acids. The spectrophotometric measurements concomitantly show that the cytochromes are turned over from a predominantly oxidized state (about 60–80 % oxidized) to a predominantly reduced state. There is, however, a rest corresponding to about 30 % of the maximal anion respiration, which is not inhibited by a far-going removal of salts and organic acids from the root tissue. This bottom level of the anion respiration can be either a real anion respiration, fed by the remaining traces of mobile anions, or it can be identical with a peroxidase respiration. It is an interesting coincidence that the hypothetical value of the peroxidase respiration, viz. about 20 % of the anion respiration, is not very far from the actually found bottom value of the anion respiration. This fact speaks in favour of the assumption that the bottom level of that fraction of the respiration in roots which is inhibited by HCN is not due to cytochrome alone but in part peroxidase.

Only cytochrome needs anions as coenzymes, because these facilitate the electron transference (LUNDEGÅRDH 1953b). In peroxidase the activity is probably not depending upon valency changes of the prosthetic iron (SUMNER & MYRBÄCK 1951, p. 428). This fact is also a very strong argument speaking against peroxidase acting in a mechanism for active salt transport. Other facts speaking against a quantitative relation between peroxidase and salt uptake are the following: (1) The anion respiration was mostly determined after the addition of 10^{-3} m. KCN; addition of HCN induces a somewhat increased inhibition in the tip zone (0–30 mm; see LUNDEGÅRDH 1949b) and this "third respiration" is possibly identical with the peroxidase respiration because the tip zone contains more peroxidase (and less cytochrome *b–dh*) than the zone above 40 mm; (2) carbon monoxide in the dark acts similar to KCN and the inhibition is reversed by light; peroxidase is not inhibited by CO; (3) there is no quantitative relation between peroxidase and salt uptake, because the latter shows a linear relation to the quantity of cytochrome *b* which in its turn varies partly opposite to the peroxidase.

c. Interactions of the flavoprotein and cytochrome systems

The higher activity of the cytochrome system probably impedes an effective competition from the side of the flavoprotein system. It can be postulated that normally the cytochromes *c* and *a* consume the entire stream of electrons from the dehydrogenase (cytochrome *dh*), even if a junction with the other system must be postulated at cytochrome *c* or at a factor between *b* and *c* (the Slater factor; SLATER 1949). This postulate is supported by the fact that the oxidation-reduction balance not only of cytochrome *b*, viz. the key of the salt transport (LUNDEGÅRDH 1953a), but also of cytochromes *c* and *a* is moving synchronously at variations of the coenzymatic activity of salt anions.

The activity of the succindehydrogenase is thus normally confined mainly to the cytochrome-cytochrome oxidase system. It is, however, quite feasible to assume that an incomplete cytochrome system consisting only of *b* and *dh* can feed a flavoprotein system with electrons provided that a junction exists, viz. *c* or the Slater factor, and that the potential of cytochrome reductase is not appreciably lower than that of cytochrome *b*. The recent observations of HACKETT & SIMON (1954) of a flavoprotein system in the spadix of *Arum*, in the absence of cytochrome oxidase cooperating with succindehydrogenase, point to the existence of such an arrangement. Only spectrophotometrical observations can reveal the presence of the dehydrogenating group of cytochromes, and such observations were omitted in the work with *Arum*.

An inhibitor which inactivates the cytochrome oxidase but largely leaves the group *b–dh* intact, is cyanide, and *b* may possibly be involved in the ground respiration. Several inhibitors are known, on the other hand, which leave *a* and *c* intact and uncouple the group *b–dh* as a source of electrons. To these belong urethane, malonate, fluoride and dinitrophenol (LUNDEGÅRDH 1952, 1953a). Under these circumstances the oxidizing activity of *a* is turned via *c* and the junction competitionally to the flavoprotein system. The result is an intensive cytochrome respiration without active uptake of salts.

The question now arises whether or not a competition is going on between the oxidase systems I and II under normal conditions. It is difficult to answer this question experimentally. But the high activity (turnover number) of the cytochrome oxidase is no doubt sufficient for the consumption of the total stream of electrons from the succindehydrogenase (via *b*) plus an additional stream of electrons from the flavoprotein system. This supposition is corroborated by the fact that under normal aerobic conditions and in the presence of salts the system *a–c* shows a lower percentage of oxidation (64–69 %) than cytochrome *b* (84 %). A similar junction between two dehydrogenase systems has been observed in animal tissue preparations (SLATER 1949, 1950), but nothing is known hitherto of the existence of an oxidase II in these tissues. In the plant tissues the oxidase II apparently takes care of a larger fraction of the capacity of the flavoprotein-dehydrogenase system (probably including Co I) if the cytochrome oxidase is put out of action.

If the junction at cytochrome *c* collects electrons from two dehydrogenase systems it can be expected that the group *a–c* will be more sensitive to a decreasing oxygen concentration than the group *b–dh*. Unpublished experiments on the displacement of the oxidation-reduction balance of the cytochromes at reduced supply of oxygen corroborate this conclusion. If the concentration of the dissolved oxygen is lowered to about $^1/_5$ of the normal saturation in contact with air ($= 0.250\ \mu\text{mol}\ O_2 \times$

lit.$^{-1}$ at 20° C) the percentage oxidation of cytochrome *c* decreases from about 64 % to about 32 %, but the oxidation of cytochrome *b* is only lowered from about 84 % to about 58 %.

d. The approximate constancy of the total aerobic respiration

Table 5 teaches us that the mean variation of the total respiration is only about one third of the variation of the ground respiration and the anion respiration, a fact also illustrated by the inverse variation of the two fractions, as shown in Figs. 12 and 14. Since the end-oxidation is branching out from intermediates of the 3-carbon type (diphosphoglyceraldehyde and pyruvic acid), the approximate constancy of the total respiration points to a controlling effect of the earlier links of the chain of glycolysis, e.g. the primary phosphorylation or the non-enzymatic addition of a second phosphate radicle to the phosphoglyceraldehyde. The fairly constant proceeding of the first links of glycolysis is, however, also regulated by the state of oxidation-reduction. At complete anaerobiosis in distilled water the rate of the glycolysis is considerably raised and corresponds to a typical Pasteur phenomenon (LUNDEGÅRDH 1949 a). A controlling and limiting effect of the earlier stages of decomposition of the carbohydrates contributes to the understanding of the comparatively slow working succindehydrogenase (cf. LUNDEGÅRDH 1953 a, 1954). The equilibria in which succinic acid are involved moreover reveal interactions with the formation and decomposition of ATP (SLATER and HOLTON 1954; unpublished experiments by the author) which may invoke a certain sluggishness.

A system alternating with the cytochrome system has been recently described by MAPSON & GODDARD (1951), viz. hydrogen from the tricarboxylic cycle, e.g. in the malate equilibrium, carried over to Co II and from here via glutathion and ascorbic acid to oxygen. Besides of glutathion reductase and ascorbic acid oxidase a flavoprotein is believed to cooperate, by means of which a side linkage to the cytochrome system may occur. A diphosphopyridine nucleotide-cytochrome *c* reductase with a flavoprotein (flavin dinucleotide) as prosthetic group has been mentioned by MAHLER *et al.* (1952). It is not identical with diaphorase. Its kinetic properties has been studied by VERNON *et al.* (1952).

That a cytochrome *c* reductase is operating in the roots is shown from the reduction of added cytochrome *c* by root homogenate in the presence of cyanide (unpublished observations). As many flavoproteins appear to have a comparatively low oxidation-reduction potential, only slightly above zero volt, the cytochrome *c* reductase will probably have some difficulties in competing with the succindehydrogenase system, because its link next to the junction is cytochrome *b* with an oxidation-reduction

Table 5. Average values and mean variation of the different fractions of respiration of wheat roots. Series of 12 experiments. Values in µmol O_2 per 10 mg dry weight and 1 hour.

Total respiration (TR)	Anion respiration (AR)	Ground respiration (GR)
2.31±0.31 (13 %)	1.25±0.40 (32 %)	1.06±0.40 (38 %)

potential of about +0.14 volt (LUNDEGÅRDH 1953a). As mentioned above, a second flavoprotein enzyme is probably carrying over protons and electrons to the oxidase II and it may be tentatively assumed that a complex of flavoprotein enzymes forms an additional junction between oxidase II and the cytochrome system.

e. The transformation of enzymes

The investigations have revealed the existence of various transformations of enzymes. Two or three categories may be distinguished:

1. The transformation of a pool of hemin into different types of hemin enzymes, viz. cytochromes or peroxidase. A competitive balance was traced between the quantities formed of cytochrome and of peroxidase. But there is also a varying relation between different cytochromes, inasmuch as the concentration of the group *b* and *dh* is varying comparatively independently of the group *c* and *a*. Of the latter group only the concentration of cytochrome *c* could be accurately determined. It was stated that the group *b–dh* varies considerably more than cytochrome *c* (and probably *a* too), a fact explaining the repeatedly observed variations in the capacity of active salt uptake. A poor development of the *b–dh* group was found to carry over more of the end-oxidation to the flavoprotein system and to the peroxidase system. The co-variation of the concentrations of flavoprotein and peroxidase supports the idea of a linkage between these systems, e.g. via H_2O_2 produced by the former and consumed by the latter.

A competitional variation of peroxidase and cytochromes *b* and *dh* is not surprising, because these three enzymes have protohematin IX as prosthetic group. The hemochromogens of cytochrome *c* and cytochrome oxidase are of different types. But even if the cytochromes *b* and *dh* show a pronounced covariation, clearly distinguished from cytochrome *c*, a more individual variation can be noticed in the case of *dh* than in the case of *b*.

Marked changes in the cytochrome content of bakers' yeast can occur under certain conditions of culture (STIER & CASTOR 1941). CHIN (1950; cf. HILL & HARTREE 1953, p. 134) observed a development of bands of the cytochromes *c* and *b* from a hemin precursor at aeration in a phosphate-glucose medium. Similarily to our experiments with roots a parallelism was shown between the development of the cytochromes and the aerobic respiration. Chin found a strong inhibition of the cytochrome transformation by DNP. Similar observations were made by EPHRUSSI & SLONIMSKY (1950) who also studied the question from the viewpoint of genetics. Because the pool of hemochromogen is commonly richly dimensioned—this is apparently the case both in roots and in yeast—the limiting reaction in the transformation to cytochrome is apparently on the side of the protein. This would explain the necessity of aeration, a fact also noticeable in the experiments with reversion of the cyanide inhibition, and of an appropriate supply of raw material for protein synthesis.

2. The transformation of distinct hemin enzymes to another member of the "family" of which an example is given by the reversal of the cyanide inhibition of the cytochrome oxidase. This transformation was interpreted as a slight modification of the apoenzyme conveying a more efficient shielding off of cyanide in its competition with oxygen. The observed results point to the existence of more than one stable molecular linkage between the hemin and the protein, the processes of formation of the enzyme probably oscillating between a number of possible configurations. Under the prevailing metabolic conditions one of the configurations is more stable than the

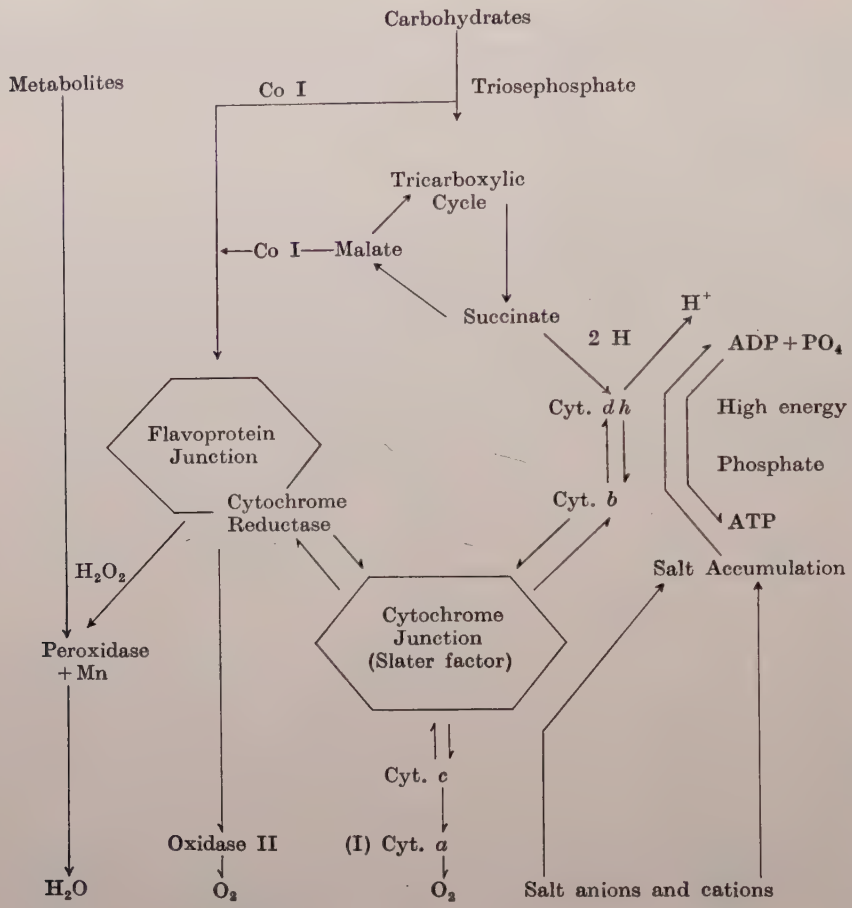

Carbohydrates
Metabolites
Co I
Triosephosphate
Tricarboxylic Cycle
Co I—Malate
Succinate
2 H
H^+
$ADP + PO_4$
Cyt. *dh*
High energy
Phosphate
ATP
Flavoprotein Junction
Cytochrome Reductase
Cyt. *b*
H_2O_2
Salt Accumulation
Cytochrome Junction (Slater factor)
Peroxidase + Mn
Cyt. *c*
Oxidase II
(I) Cyt. *a*
H_2O
O_2
O_2
Salt anions and cations

Fig. 15. Scheme of the oxidative enzyme systems in roots of wheat. The pathways of the protons are omitted in the scheme. Half arrows indicate electrons, full arrows molecules or ions.

others and will then take over most of the activity. Under changed conditions, e.g. in the presence of cyanide, another configuration is more stable. The idea of such an intracellular enzymatic adaptation is not startling, because it is known that even more deep-going variations occur in the composition of single enzymes, including combination of one and the same prosthetic group with different apoenzymes which even may cause changes in the absorption spectrum. The question of shielding off of the sensitivity to cyanide and CO has been discussed in publications by DARBY & GODDARD (1950) and HILL & HARTREE (1953).

3. In addition to the two categories mentioned, which illustrate competitional linkages between an identical prosthetic group and different proteins or different couplings to one and the same protein, a balance was observed, too, between two different types of enzymes, viz. flavoproteins and cytochromes. To elucidate the possible bearing of such a balance it may be mentioned that according to recent investiga-

tions (see NEUBERGER & SCOTT 1953) protoporphyrin is synthesized by condensation of eight molecules of glycine with eight molecules of a compound derivable from α-oxoglutarate, possibly aminolaevulinic acid. The synthesis of hemins is thus intimately connected with the carbohydrate metabolism and a competitive variation of two different enzyme systems may be brought back to some metabolite common to the two chains of reactions which lead to the enzymes.

Summary

The enzyme systems carrying on aerobic respiration in roots were studied by means of a sensitive and rapid spectrophotometric technique and measurements of the oxygen consumption.

Three enzyme systems are cooperating in the normal aerobic respiration: 1. The cytochrome system (cytochromes *a*, *c*, *b*, and *dh* + succindehydrogenase), 2. a flavoprotein system (probably cooperating with coenzyme), 3. a peroxidase system (probably cooperating with flavoprotein and possibly Mn^{++}). System 1 conducts the anion respiration and the active transport of salts, system 2 conducts the ground respiration (= a fraction of the total respiration highly resistent to cyanide, azide and CO), system 3 conducts a minor part of that fraction of the total respiration which is inhibited by HCN and is possibly identical with the "third respiration" in the downmost part of the roots (LUNDEGÅRDH 1949b). The turnover numbers per minute of the three systems are 170, 80, and about 3–5, as referred to cytochrome *b*, flavoprotein, and peroxidase respectively.

The separate enzyme systems are probably starting out from different points of the glycolytical chain or tricarboxylic cycle but are competitively linked together at "junctions" or "factors", which may be studied by means of inhibitors. The oxidase of the second system ("oxidase II") is still unidentified, but different possibilities are discussed. The "peroxidase respiration" was determined in horse radish and the results hypothetically transferred to the wheat roots, in which this enzyme is present in a higher concentration than cytochrome, particularly in the downmost portion.

The quantitative relations between the enzymes are changed with the external conditions. In the cytochrome system two groups can be distinguished, viz. *a–c* and *b–dh*, of which the latter is more varying than the former. It varies inversely to the flavoprotein system and to the concentration of peroxidase.

With regard to observations of a reversal of inhibition of the cytochrome system by cyanide and azide the possibility of a transformation of enzymes within a "family of enzymes" was discussed. It was concluded that oxygen and cyanide are competing at the surface of the cytochrome oxidase and that small changes in the configuration of the protein may strongly impede the formation of an enzyme-CN complex.

REFERENCES

BEEVERS, H., Bioch. J. *49*, 642 (1951).
CHANCE, B., Nature *161*, 914 (1948).
DARBY, R. T. and GODDARD, D. R., Physiol. Plantarum *3*, 435 (1950).
EPHRUSSI, B. and SLONIMSKY, P. P., Compt. rend. *230*, 685 (1950).
GALSTON, A. W. and BAKER, R. S., Amer. J. Bot. *38*, 190 (1951).
HACKET, D. P. and SIMON, E. W., Nature *173*, 162 (1954).
HILL, R. and HARTREE, E. F., Ann. Review Plant Physiol. *4*, 115 (1953).

HORECKER, B. L., J. Biol. Chem. *183*, 593 (1950).
JAMES, W. O., Ann. Review Plant Physiol. *4*, 59 (1953).
JAMES, W. O. and BEEVERS, H., New Phytologist *49*, 353 (1950).
KEILIN, D. and HARTREE, E. F., Biochem. J. *49*, 88 (1951).
KEILIN, H., Nature *172*, 390 (1953).
KENTEN, R. H. and MANN, P. J. G., Biochem. J. *45*, 255 (1949); *46*, 67 (1950); *53*, 498 (1953).
LEMBERG, R. and LEGGE, J. W., Hematin Compounds and Bile Pigments. New York 1949.
LUNDEGÅRDH, H., Biochem. Zeitschr. *298*, 51 (1938).
—— Planta *29*, 419 (1939).
—— Ann. Agric. Coll. Sweden *8*, 233 (1940).
—— Protoplasma *35*, 548 (1941).
—— Ark. f. Bot., Stockholm *31 A*, Nr. 2 (1943).
—— Ark. f. Bot., Stockholm *32 A*, Nr. 12 (1945).
—— Ann. Agric. Coll. Sweden *16*, 339 (1949a).
—— Ann. Agric. Coll. Sweden *16*, 372 (1949b).
—— Physiologia Plantarum *2*, 388 (1949c).
—— Physiologia Plantarum *3*, 103 (1950a).
—— Nature *165*, 513 (1950b).
—— Arkiv f. Kemi (Swed. Acad. Sc.) *3*, 69, 469 (1951).
—— Nature *169*, 1088 (1952).
—— Arkiv f. Kemi (Swed. Ac. Sc.) *5*, 97 (1953a).
—— Nature *171*, 477 (1953b).
—— Nature *171*, 521 (1953c).
—— Nature *172*, 303 (1953d).
—— Nature *173*, 939 (1954).
LUNDEGÅRDH, H. and BURSTRÖM, H., Biochem. Zeitschr. *261*, 235 (1933).
—— Biochem. Zeitschr. *277*, 223 (1935).
LUNDEGÅRDH, H. and STENLID, G., Ark. f. Bot., Stockholm *31 A*, Nr. 10 (1944).
MAHLER, H. R. *et al.*, J. Biol. Chem. *199*, 585 (1952).
MAPSON, L. W. and GODDARD, D., Biochem. J. *49*, 592 (1951).
NEUBERGER, A. and SCOTT, J. J., Nature *172*, 1093 (1953).
ROBERTSON, R. N., Ann. Review Plant Physiol. *2*, 1 (1951).
SLATER, E. C., Nature *166*, 982 (1950).
—— Biochem. J. *45*, 14 (1949); *46*, 484 (1950).
SLATER, E. C. and HOLTON, F. A., Biochem. J. *56*, 28 (1954).
STENLID, G., Physiologia Plantarum *1*, 185 (1948).
—— Physiologia Plantarum *2*, 350 (1943).
—— Physiologia Plantarum *3*, 197 (1950).
STIER, T. J. B. and CASTOR, J. G. B., J. Gener. Physiol. *25*, 229 (1941).
SUMNER, J. B. and MYRBÄCK, K., 1951, The Enzymes, Vol. II, Part 1, New York.
SUTTER, ERIKA, Experientia *6*, 264 (1950).
VERNON, L. P. *et al.*, J. Biol. Chem. *199*, 599.
WARBURG, O., Die Naturwissenschaften *22*, 441 (1934).
WAYGOOD, E. R., Canad. J. Research *C 28*, 7 (1950).

Tryckt den 17 november 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 51

Communicated 2 June 1954 by ARNE FREDGA and GUNNAR HÄGG

Stereochemical studies on dicarboxylic acids

I. The infrared absorption of some α, α′-dimethylsubstituted acids

By LENNART SCHOTTE and ANDREAS ROSENBERG

With 9 figures in the text

In connection with a study of the steric isomers of certain derivatives of aliphatic dicarboxylic acids we have investigated the infrared absorption of the different steric forms of some α,α′-dimethylsubstituted acids. A short communication about the most interesting results has recently been published (1). Although previous work on this subject has been scarce, two papers by ELIEL and KOFRON (2) and DUNCANSON (3) drew our attention to the question whether differences between steric isomers could be easily detected and explained using infrared methods. It has, however, not been our intention to study any presumable small differences between (+)- and (−)-forms, e.g. as observed by GORE and PETERSEN (4). Such effects are difficult to explain from the theoretical point of view and have been attributed to impurities or to polymorphism. GILMAN gives in his treatise (5) an explanation of the fact that identity of the spectra of enantiomorphs in vapor, liquid or solution is expected to exist. The vibrational energy levels are determined by the potential function of the molecule, which is a mathematical expression giving the potential energy as a function of the displacement of the nuclei from their equilibrium positions. If now two molecules are considered, completely identical except for being mirror images of each other, it may be assumed that their potential functions must also be mirror images. This means that the mathematical expressions for the two potential functions can be made identical by proper choice of the positive direction of the coördinate axis perpendicular to the mirror plane, which choice, however, is quite arbitrary and cannot change the physical state of the molecule. Since the potential functions of the two isomers can thus be made identical in this manner, it follows that their vibrational energy levels must be identical and hence their vibrational spectra. An experimental verification of this interpretation by GILMAN is given by ELIEL and KOFRON (2) in their examination of the infrared spectra of the optical isomers of the acid phthalic ester of *p*-ethylphenyl-methyl-carbinol. Their results show that the active and racemic forms have identical spectra in chloroform solution, whereas those of the crystalline forms, taken as nujol mulls, exhibit significant differences. The absence of free OH stretching frequency and at the same time a shift in the main carbonyl frequency was most interesting. From the fact that this effect was not found for the corresponding ester without substituent in the phenyl group of the alcohol component, the authors came to the conclusion that changes in the main carboxylic frequencies may be expected only when the (+)- and (−)-forms alone cannot dimerize.

The existence of differences in the infrared spectra of optically active and racemic compounds as observed above is interesting from the structural point of view, but it would be far more interesting to study the behaviour of active and racemic form in solution. This work must, however, be carried out on substances, where the tendency to form racemic compounds is assumed to be especially high. In such cases it may, in spite of the negative results by ELIEL and KOFRON, be possible to obtain experimental evidence for the presumable existence of racemic compounds in solution, the proof of which in general seems very difficult to obtain. From the investigations of FREDGA (6) on the steric relationships of optically active acids it can be seen that the antipodes of α,α′-dimethylglutaric acid give a melting point curve of a rather exceptional type. It obviously reveals the existence of a racemic compound of great stability, melting as much as 61° higher than the active forms. It thus seemed suitable to the authors to devote special interest to the optically active and racemic form of this acid, as the solubility conditions do allow an examination of both the forms in a solvent such as chloroform. The results of this study have already been briefly reported in (1). The difference observed was small, but in spite of careful studies with different concentrations it could not be ascribed to the disturbing influence of the solvent chloroform, which has an absorption band in the near region. Further investigations of other acids with great differences in the melting points, such as disulphide-di-α-propionic acid (dithio-dilactic acid) (7) and methylene-di-α-mercaptopropionic acid (methylene bis-thiolactic acid) (8), were unsuccessful due to complete insolubility in solvents suitable for infrared absorption measurements. However, it may be worth mentioning that in such a good solvent as N,N-dimethyl formamide both (+)- and racemic form of α,α′-dimethylglutaric acid show essential differences compared with the chloroform solutions. The broad maximum at 10.7 μ (935 cm^{-1}) has now been clearly divided into three bands at 10.40, 10.70 and 11.05 μ respectively. This behaviour is certainly explained by an interaction of complex nature with the solvent, and it is quite interesting to note that the division of this maximum is analogous to the double peak of the racemic compound in chloroform solution. Thus some evidence for the existence of racemic compounds in solution has been presented, but the definite confirmation must wait until further proof is available.

The main part of this work has, however, been devoted to the study of the spectra of the diastereoisomers of α,α′-dimethylsubstituted succinic, glutaric and adipic acids. The influence of the spatial distribution of substituent groups on the infrared spectra is frequently a question of interest, and it has been observed that the spectra will, in general, differ from one another, no matter what the physical state may be. Of work previously done, DUNCANSON's study of variations produced by sublimation in the infrared spectra of some mono- and disubstituted succinic acids (3) is nearest to the subject of our investigation. Unfortunately most of the substances that DUNCANSON has studied belong to the monosubstituted series, and as at the same time the object of his investigation was quite different from our's no direct comparison of the experimental material could be made. DUNCANSON arrives at the conclusion that the main difference between the mono- and disubstituted succinic acids is due to steric hindrance, which is specially pronounced for the α,α′-disubstituted compounds. As will be shown later the same conclusions can be drawn from our experimental material concerning the succinic acid derivatives.

We have examined the infrared spectra of all the stereoisomeric forms of α,α′-dimethylsuccinic acid, viz. the (+)-, (−)-, racemic and meso forms. Figures 1–4 show the spectra of these compounds in the solid state. The wavelength of the absorption bands can be seen from Table 1. A comparison of the spectra of the (+)- and (−)-forms of α,α′-dimethylsuccinic acid shows that the infrared absorptions of the enantiomorphs are quite identical. It can thus be concluded that their crystal structures are completely similar. Concerning the spectra of the racemic form of the same acid it can first of all be established that it is slightly less complicated than that of the active form, even if the latter has a few bands more. In the hydrogen bond stretching region, as revealed by sodium chloride prism, the differences are negligible. In the double bond region the carbonyl stretching peak at 5.85 μ for the active form with a shoulder at 5.78 μ has in the racemic form given place to two distinct maxima, one at 5.77 μ and the other at 5.87 μ. From the rest of the spectrum it can be seen that most of the bands have changed in position. The differences are especially great in the region between 8 and 9 μ, where the bands connected with the oxygen containing groups are situated. The broad band at 10.7 μ, on the other hand, commonly ascribed to out of plane bendings of OH, has in the racemic form been slightly shifted to 10.8 μ.

As might be expected the most interesting differences in the infrared absorption curves could be observed by comparing the meso and racemic forms of the α,α′-dimethylsuccinic acid. The general picture of the spectrum of the meso isomer resembles more closely that of the optically active form than that of the racemic one. The hydrogen bond stretching region is identical with those of the other forms. But in the double bond region all tendencies to form a shoulder at 5.78 μ have vanished, and the carbonyl stretchings show a single peak at 5.87 μ. In the area

Table 1. The infrared absorption bands of α,α′-dimethylsuccinic acids. b = broad. vs = very strong. s = strong. m = medium. w = weak. sh = shoulder.

(+) − and (−)-Forms			Racemic form			Meso form		
Wave-length in μ	Wave-number in cm⁻¹	Character of the band	Wave-length in μ	Wave-number in cm⁻¹	Character of the band	Wave-length in μ	Wave-number in cm⁻¹	Character of the band
3.4	2941	sb	3.4	2941	sb	3.4	2941	sb
3.80	2631	w	3.82	2617	w	3.82	2617	w
5.78	1730	sh	5.77	1733	m	3.95	2531	sh
5.85	1709	vs	5.87	1707	s	4.25	2352	sh
6.83	1463	s	6.84	1461	m	5.87	1707	vs
7.01	1426	s	7.07	1414	s	6.84	1461	m
7.40	1351	sh	7.47	1338	m	7.02	1424	s
7.57	1321	w	7.95	1257	m	7.59	1317	s
7.77	1287	s	8.05	1240	s	7.80	1282	m
7.89	1267	w	8.67	1153	m	8.25	1212	m
8.23	1215	s	8.87	1127	m	8.34	1199	s
8.61	1161	w	9.37	1067	m	8.50	1176	sh
9.10	1098	sh	10.8	925	sb	9.22	1084	s
9.26	1079	m	11.82	846	m	9.47	1056	w
9.45	1058	m	12.05	830	w	9.90	1010	sh
9.80	1020	w	13.43	744	w	10.25	976	m
10.40	962	sh				10.65	943	sb
10.7	935	sb				11.67	857	m
11.75	852	m						
12.15	824	w						
14.95	668	m						

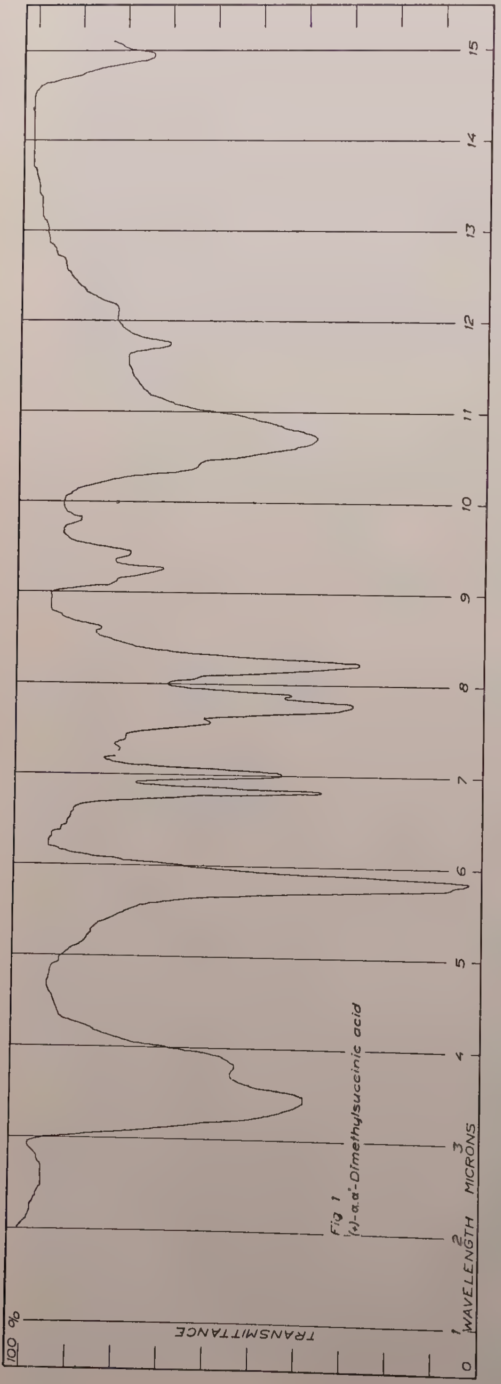


Fig 1
(+)-α,α'-Dimethylsuccinic acid

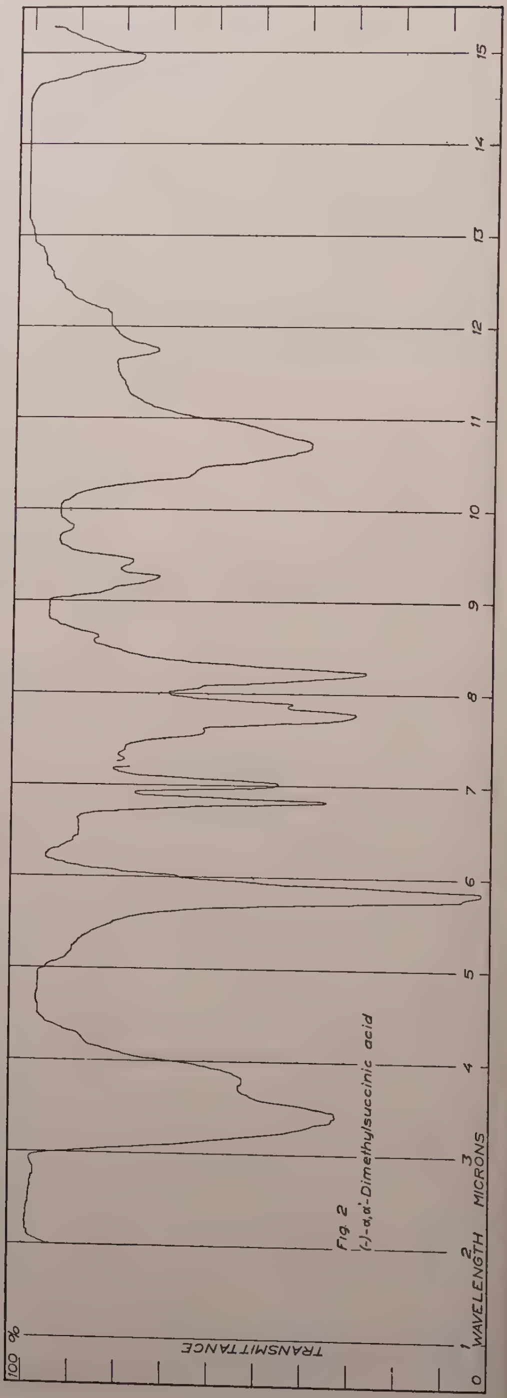


Fig 2
(−)-α,α'-Dimethylsuccinic acid

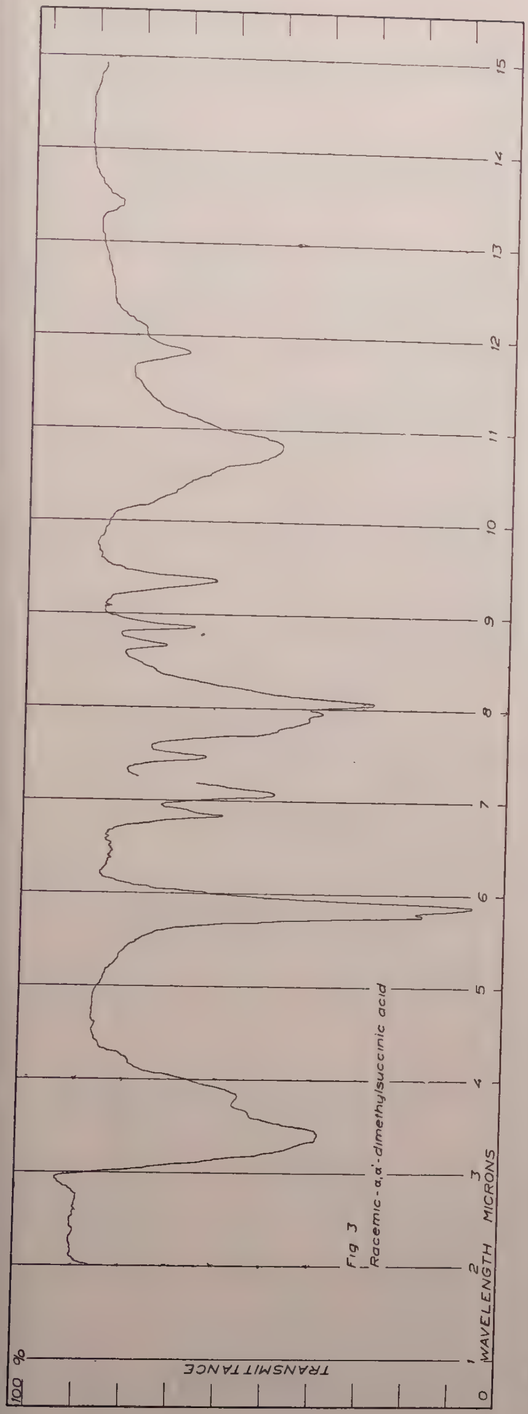


Fig 3
Racemic-α,α'-dimethylsuccinic acid

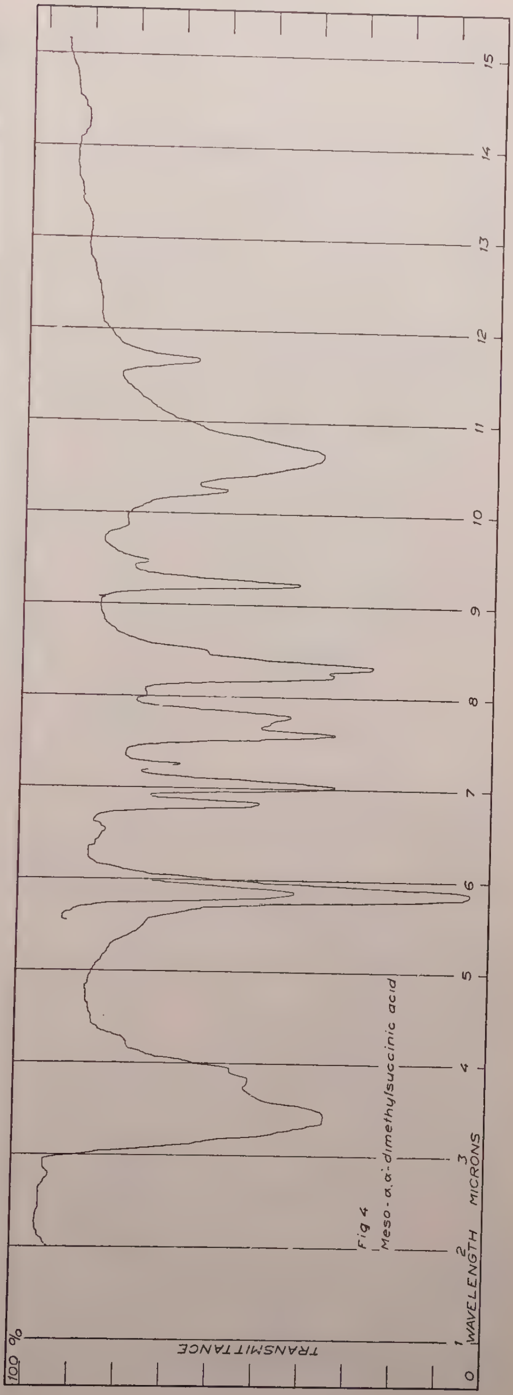


Fig 4
Meso-α,α'-dimethylsuccinic acid

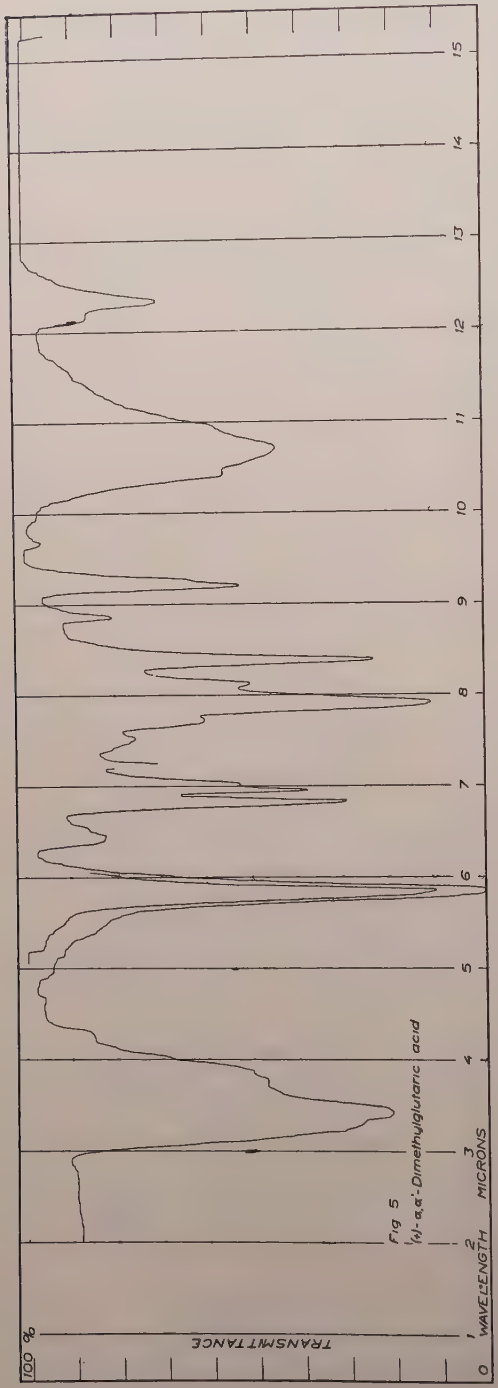


Fig 5
(+)-α,α'-Dimethylglutaric acid

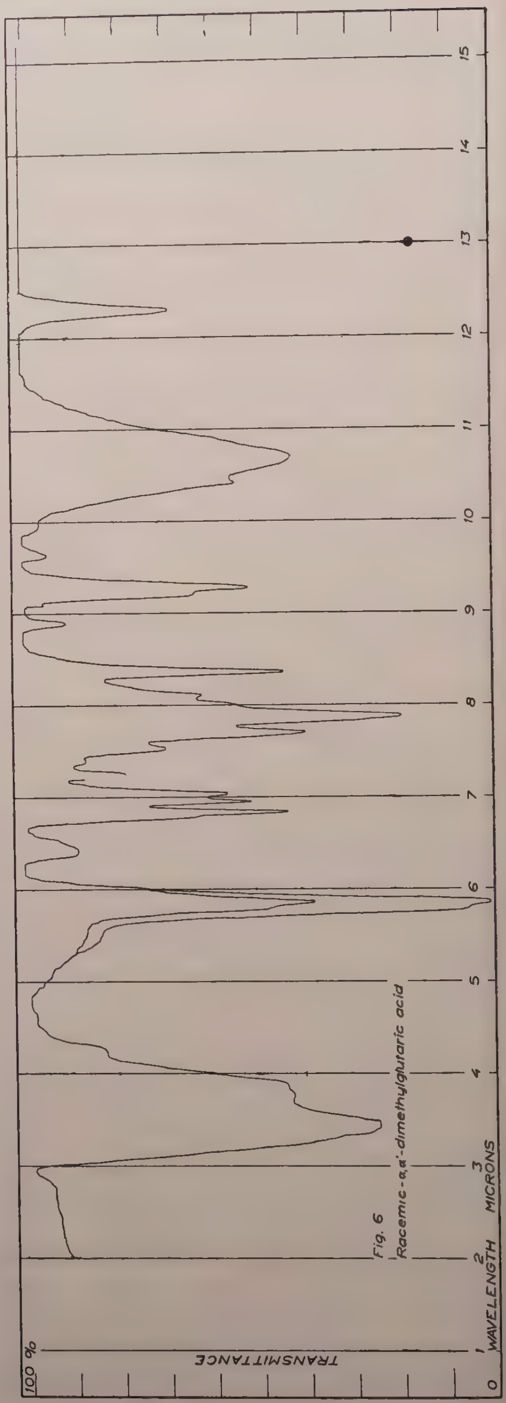


Fig. 6
Racemic-α,α'-dimethylglutaric acid

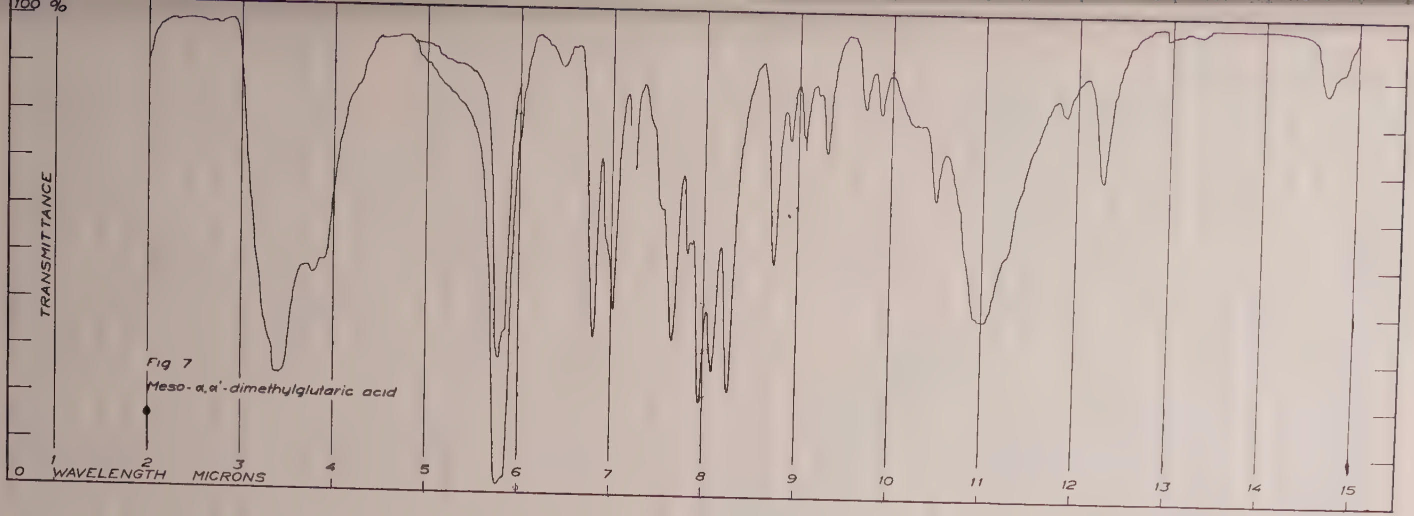


Table 2. The infrared absorption bands of α,α′-dimethylglutaric acids. For abbreviations see Table 1.

(+)-Form			Racemic form			Meso form		
Wave-length in μ	Wave-number in cm⁻¹	Char-acter of the band	Wave-length in μ	Wave-number in cm⁻¹	Char-acter of the band	Wave-length in μ	Wave-number in cm⁻¹	Char-acter of the band
3.25	3076	sh	3.4	2941	s b	3.4	2941	s b
3.4	2941	s b	3.75	2666	w	3.76	2659	w
3.75	2666	sh	3.90	2564	sh	3.90	2564	sh
3.90	2564	sh	4.25	2352	sh	5.79	1727	vs
4.25	2352	sh	5.80	1724	sh	5.85	1709	sh
5.87	1703	vs	5.87	1703	vs	6.45	1550	w b
6.45	1550	w b	6.45	1550	m b	6.82	1466	s
6.84	1461	s	6.85	1459	s	7.03	1422	s
6.97	1434	s	6.96	1436	s	7.55	1324	sh
7.02	1424	sh	7.05	1418	m	7.66	1305	s
7.52	1329	w	7.40	1351	w	7.83	1277	m
7.72	1295	w	7.52	1329	w	7.87	1270	w
7.91	1264	s	7.70	1298	m	7.96	1256	s
8.12	1231	m	7.88	1269	s	8.10	1234	s
8.40	1190	s	8.10	1234	w	8.27	1209	s
8.85	1129	m	8.36	1196	s	8.75	1142	s
9.20	1086	s	8.90	1123	m	8.92	1121	m
9.70	1030	w	9.20	1086	sh	9.07	1102	m
10.45	957	sh	9.28	1077	s	9.32	1072	m
10.7	935	s b	9.65	1036	w	9.72	1028	m
12.15	824	sh	10.0	1000	sh	9.90	1010	m
12.35	809	m	10.41	961	w	10.25	976	sh
			10.70	935	s	10.50	952	m
			12.30	813	m	11.0	909	s b
						11.87	843	w
						12.30	813	s
						14.70	680	m

around 8 μ the changes as compared to the active form are distinct but small, contrary to the behaviour of the racemic form, which changes completely in this part of the curve. The OH-band shows a slight shift to 10.65 μ for the meso compound. The most interesting result obtained by comparing the absorption curves of these stereoisomeric succinic acid derivatives is the observation that the carbonyl band in the racemic form has split to a double peak, whereas the meso compound shows a single one. The presence of this double peak in the racemic form is probably due to symmetrical and antisymmetrical modes of vibrations involving the carbonyl stretching, as DUNCANSON (3) has found similar effects for the unsubstituted malonic and succinic acids. This comparison seems justified, as the spatial distribution of the methyl groups in the active and racemic isomers of α,α′-dimethylsuccinic acid is such that the free rotation around the carbon-carbon bond does not seem to be limited to any extent worth mentioning. A consideration of molecular models shows, on the other hand, that owing to the steric influence of the two adjacent methyl groups the meso form must be ascribed some differing properties, as the free rotation seems to be appreciably reduced. In all probability such an effect means that the most favoured structure for the meso compound is one, in which the carboxylic groups in the crystalline state are situated as far from each other as possible. At the same time the small change in the position of the infrared absorption band at 10.7 μ can be interpreted as indicating that the distances between carbonyl and hydroxyl groups in the crystal lattice are of approximately the same magnitude as those of the racemic and optically active form (5).

In the case of such an interpretation it seems natural to question whether it could be possible to observe similar differences in the spectra of other α,α′-disubstituted succinic acids, where the substituents are larger than the methyl groups. In a previous publication (1) we have already shown that the α,α′-dibromosuccinic acid diastereoisomers exhibit the same features as those discussed above, yet in an even more pronounced way in good agreement with the proposed interpretation. A thorough investigation of the homologous series of the α,α′-dibromosubstituted aliphatic dicarboxylic acids will be reported later, when the question of mutual conversion between the diastereoisomers in solution has been further elucidated. Naturally it would have been of interest to compare these substances even in suitable solvents, but unfortunately this could not be achieved owing to the complete insolubility of the meso forms. Some indications that the peaks were not caused by any impurities could, however, be found for the optically inactive isomers of the α,α′-dibromosuccinic acid in dioxane solution, where the spectra showed well defined single peaks.

The next homologue in the α,α′-dimethylsubstituted dicarboxylic acid series to be examined was α,α′-dimethylglutaric acid. Figures 5–7 show the infrared absorption curves of the (+)-, racemic and meso forms in the crystalline state, and the wavelength of the bands are given in Table 2. It is interesting to note here that the spectra of the active and racemic form show only extremely small differences in the form of slight shifts in the position and intensity of certain peaks. The behaviour does not at all correspond to the above described effect for α,α′-dimethylsuccinic acid. It is thus obvious that the length of the carbon chain between the two carboxylic groups renders it possible for the active form of α,α′-dimethylglutaric acid to crystallize in a lattice in certain respects similar to that of the racemic compound, whereas this possibility seems excluded for the succinic acid derivative from molecular model studies. This can possibly be connected with the results arrived at by MORRISON and ROBERTSON (9 and 10) in their X-ray studies on the crystal and molecular structure of the unsubstituted acids. Further, our results show that the theory put forward by LECOMTE (3) about the high symmetry of racemates, based upon the establishment of far simpler spectra for the racemic compounds than for the active, cannot at all be regarded as general.

If we now compare the racemic and meso forms of α,α′-dimethylglutaric acid it can be seen that in spite of the similarity of the hydrogen bond stretching region the rest of the absorption spectra show considerable differences. The racemic form has the carbonyl peak at 5.87 μ with a slight shoulder at 5.80 μ. The meso form, on the other hand, has the corresponding peak at 5.79 μ with a shoulder at 5.85 μ. At the same time the OH band at 10.7 μ in the racemic compound has been shifted in the meso isomer to 11.0 μ. These results are best interpreted by assuming that the meso configuration favours a crystal structure, where the distances between the oxygen atoms of carbonyl groups are longer. This means that the crystal lattice of the meso form is not so closely packed as that of the racemic compound. From the spectra of the meso and racemic form in chloroform solution it can be concluded that the shifts of the carbonyl stretching are not only due to intermolecular hydrogen bonds: the racemic form shows a single carbonyl peak at 5.84 μ, whilst the corresponding peak in the meso compound is shifted to 5.79 μ. This shift observed at different concentrations must be due to an intramolecular effect of the carboxylic groups. Preliminary studies have further shown that the α,α′-dibromoglutaric acids exhibit shifts of the same kind and direction.

The shift in the absorption frequencies of the diastereoisomers of α,α′-dimethyladipic acid is too small to be determined with certainty, as can be seen from Figures 8–9 and Table 3. This fact is obviously due to the high degree of free rotation and to the flexibility, made possible by the length of the carbon chain.

The experimental results have already been shortly discussed and interpreted for the special homologues of the α,α′-dimethylsubstituted dicarboxylic acids. It is,

Table 3. The infrared absorption bands of α,α′-dimethyladipic acids. For abbreviations see Table 1.

Racemic form			Meso form		
Wavelength in μ	Wavenumber in cm^{-1}	Character of the band	Wavelength in μ	Wavenumber in cm $^{-1}$	Character of the band
3.40	2941	s	3.40	2941	s
3.75	2666	sh	3.75	2666	sh
5.86	1706	s	5.86	1706	s
6.81	1468	m	6.81	1468	m
7.06	1416	m	7.06	1416	m
7.50	1333	mb	7.45	1342	w
7.70	1298	m	7.55	1324	w
7.85	1273	m	7.72	1295	m
8.10	1234	s	8.00	1250	s
8.37	1194	m	8.50	1176	s
8.72	1146	w	9.05	1104	m
8.92	1121	w	9.25	1081	w
9.25	1081	m	9.85	1015	w
9.80	1020	w	11.0	909	sb
10.85	925	sb	12.75	784	mb
12.8	781	mb			

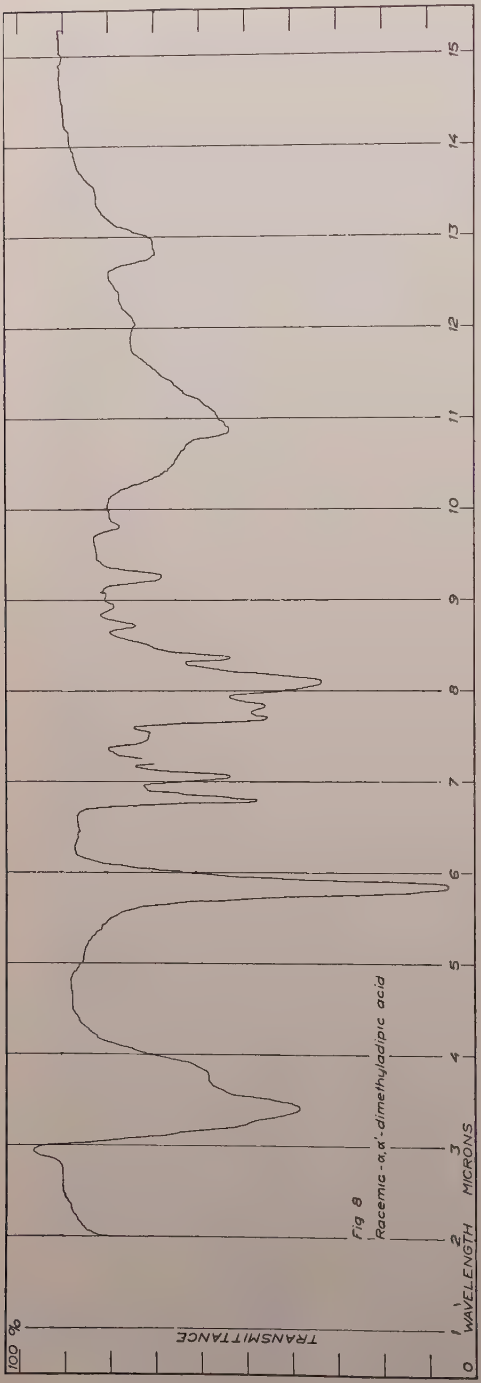


Fig 8 Racemic-α,α'-dimethyladipic acid

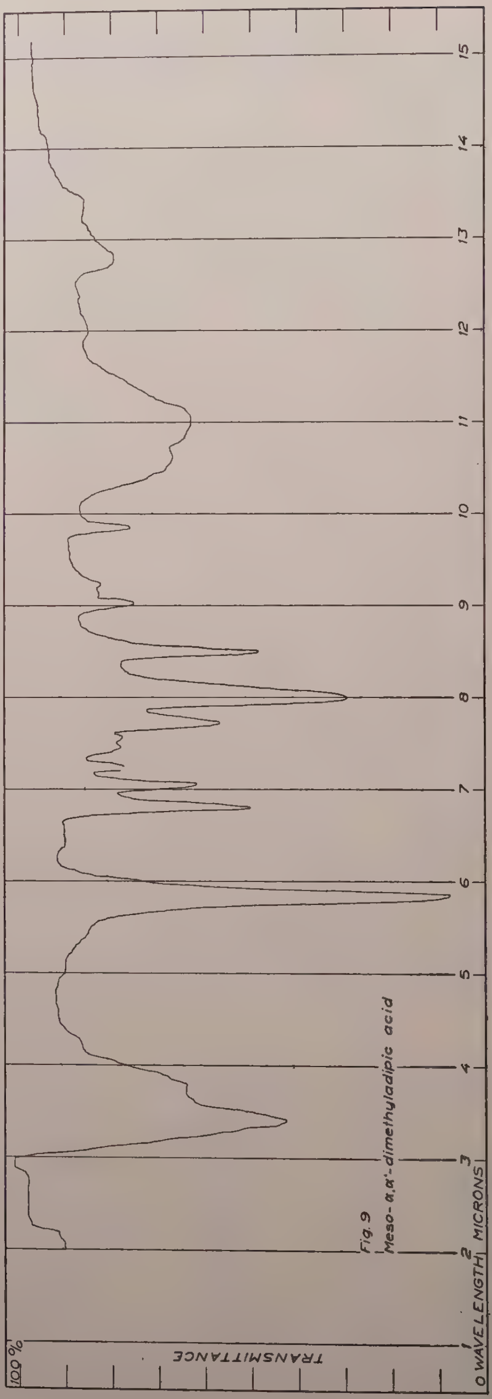


Fig. 9 Meso-α,α'-dimethyladipic acid

however, of great importance to consider the material from a more general point of view as some interesting conclusions can obviously be drawn. Thus our results throw new light upon the assumption of DUNCANSON (3) that heavy substituents, such as methyl and chlorine, should weaken the coupling between the two modes of vibration involving the carbonyl stretching. We have, on the contrary, found that such a substitution at the α,α′-positions of succinic acids favours, in the case of racemic forms, a clear splitting of the carbonyl bands into double peaks. It seems therefore reasonable to consider this effect on the infrared absorption bands as due more to the spatial distribution of the substituents than to the individual type of the different groups.

From our experimental material and from the foregoing it is obvious that the differences between the spectra of the diastereoisomers are not constant throughout the homologous series but decrease with the length of the carbon chain. According to GILMAN (5) the nature of the differences in the spectra of diastereoisomers cannot be predicted, and they are supposed to be small in most cases. However, our investigation on the dicarboxylic acid derivatives has indicated that where differences exist, the carbonyl frequency of the racemic form is shifted to longer wavelength. Further it will be noticed that the behaviour of the main carbonyl absorption band seems to be characteristic for the stereoisomeric forms of acids, differing only in the substituents, derived from the same dicarboxylic acid. Although it should be observed that in this investigation the substituents mentioned are of approximately the same type, this conclusion may possibly prove useful for stereochemical purposes.

Experimental

A Perkin Elmer model 21 spectrophotometer equipped with sodium chloride prism was used for the infrared absorption measurements. The solid samples of the acids were examined as solid pressed potassium bromide plates according to the method described by SCHIEDT and REINWEIN (11). The calibration of the absorption curves, linear in wavelength, is exact up to $\pm 0.01\ \mu$.

All the absorption curves (Figures 1–9) are discontinous at 7.24 μ due to a sharp absorption band caused, according to MECKE (12), by nitrogen impurities in the potassium bromide.

The α, α′-dimethylsubstituted dicarboxylic acids investigated

(+)-α,α′-Dimethylsuccinic acid. M.p. 134° (preheated apparatus) (13). *Anal.* Subst., 15.20 mg: 9.92 ml of 0.0211–N NaOH. Equ. wt. Calc. 73.1; Found 72.6.

(−)-α,α′-Dimethylsuccinic acid. M.p. 133°–134° (preheated apparatus) (13). *Anal.* Subst., 11.12 mg: 7.14 ml of 0.0211–N NaOH. Equ. wt. Calc. 73.1; Found 73.8.

Racemic-α,α′-dimethylsuccinic acid. M.p. 128°–129°. (13 and 14). The substance used was prepared from the enantiomorphs.

Meso-α,α′-dimethylsuccinic acid. M.p. 197°–199° (d.). The melting point depends on the rate of heating. The substance was repeatedly recrystallized from water (13 and 14). *Anal.* Subst., 46.97 mg: 7.28 ml of 0.0879–N NaOH. Equ. wt. Calc. 73.1; Found 73.4.

(+)-α,α′-Dimethylglutaric acid. M.p. 79°. (15, 16 and 17.) *Anal.* Subst., 16.70 mg: 9.82 ml of 0.0211–N NaOH. Equ. wt. Calc. 80.1; Found 80.6.

Racemic-α,α′-dimethylglutaric acid. M.p. 140°–141°. (15, 16 and 17.) The substance used was prepared from the enantiomorphs.

Meso-α,α′-dimethylglutaric acid. M.p. 127°–128°. (15 and 18.) *Anal.* Subst., 51.55 mg: 7.20 ml of 0.0898–N NaOH. Equ. wt. Calc. 80.1; Found 79.7.

Racemic-α,α′-dimethyladipic acid. M.p. 75°–77°. A mixture of the diastereoisomers of α,α′-dimethyladipic acid was extracted several times with boiling benzene. The benzene solution deposited on cooling a low-melting acid. The procedure was repeated twice with this substance and the product obtained eventually recrystallized three times from water. (19, 20 and 21.) *Anal.* Subst., 50.70 mg: 6.48 ml of 0.0898–N NaOH. Equ. wt. Calc. 87.1; Found 87.1.

Meso-α,α′-dimethyladipic acid. M.p. 140°–142°. The substance was obtained by repeated recrystallizations from water of the benzene-insoluble fraction in the separation described above for the racemic isomer. (19, 20 and 21.) *Anal.* Subst., 16.43 mg: 8.88 ml of 0.0211–N NaOH. Equ. wt. Calc. 87.1; Found 87.7.

Summary

The infrared absorption curves of stereoisomers of α,α′-dimethylsubstituted succinic, glutaric and adipic acid have been studied.

The differences observed have been discussed from the structural point of view.

Some evidence for the existence of racemic compounds in solution is presented.

Certain regularities have been pointed out in the differences between the spectra of the meso and racemic forms of the succinic and glutaric acid derivatives.

ACKNOWLEDGEMENTS

The authors want to express their gratitude to Professor Arne Fredga and Professor Arne Tiselius for stimulating interest. The substances were kindly placed at our disposal by Professor Fredga.

The investigation has partly been financially supported by the Rockefeller and Nobel Foundations.

Laboratory of Organic Chemistry, Chemical Institute and Institute of Biochemistry, University of Uppsala, May 1954.

REFERENCES

1. A. Rosenberg and L. Schotte, Acta Chem. Scand., *8*, 867 (1954).
2. E. L. Eliel and J. T. Kofron, J. Am. Chem. Soc. *75*, 4585 (1953).
3. L. A. Duncanson, J. Chem. Soc. *1952*, 1753.
4. R. C. Gore and E. M. Petersen, Ann. N.Y. Acad. Sci. *51*, 924 (1949).
5. H. Gilman, Organic Chemistry. An Advanced Treatise, vol. III, p. 136, New York (1953).
6. A. Fredga, Arkiv Kemi, Mineral., Geol. *24 A* (1947) nr 32.
7. A. Fredga, Arkiv Kemi, Mineral., Geol. *12 A* (1937) nr. 13.
8. A. Fredga, Arkiv Kemi, Mineral., Geol. *12 A* (1937) nr. 15.
9. J. D. Morrison and J. M. Robertson, J. Chem. Soc. *1949*, 980.
10. J. D. Morrison and J. M. Robertson, J. Chem. Soc. *1949*, 1001.
11. U. Schiedt and H. Reinwein, Z. Naturforsch *7 b*, 270 (1952).
12. R. Mecke, Private communication to one of the authors (A.R.).
13. E. Berner and R. Leonardsen, Ann. *538*, 1 (1939).
14. W. A. Bone and C. H. G. Sprankling, J. Chem. Soc. *75*, 852 (1899).

15. E. Möller, Ber. *43*, 3250 (1910).
16. E. Möller, Optiskt aktiva α,α′-dimetylglutarsyror. Diss. Lund (1918).
17. A. Fredga, Arkiv Kemi, Mineral., Geol. *14 B* (1940) nr. 12.
18. A. Fredga, Arkiv Kemi, Mineral., Geol. *18 B* (1945) nr. 4.
19. N. A. Noyes and L. P. Kyriakides, J. Am. Chem. Soc. *32*, 1057 (1910).
20. N. Zelinsky, Ber. *24*, 3997 (1891).
21. J. H. Brewster, J. Am. Chem. Soc. *73*, 366 (1951).

Tryckt den 12 november 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 52

Communicated 2 June 1954 by Arne Fredga

The diastereomers of 2,6-tetrahydrothiapyrandicarboxylic acid

By Lennart Schotte

With 2 figures in the text

While searching for intermediates which might prove useful in the synthesis of certain thiapyran derivatives Fehnel and Oppenlander (1) prepared and investigated some symmetrical 2,6-disubstituted tetrahydrothiapyrans. Special interest was devoted to the study of *cis*-(*meso*)-2,6-tetrahydrothiapyrandicarboxylic acid, which was obtained from sodium sulphide and the sodium salt of the high melting form of α,α'-dibromopimelic acid in water solution. This method implies an application of the general procedure for the preparation of saturated cyclic sulphides (2). In the cyclizations in question the starting materials are α, α'-disubstituted halogeno, mostly bromo, derivatives of aliphatic dicarboxylic acids. Fredga has shown for the thiophane series (2) that the reactions proceed without any inversions at the asymmetric centres. Thus it seems justified to consider this statement as general, provided that structural conditions do not cause anything else (3). The meso dihalides should then lead to cis products, while the racemic dihalides should furnish the trans isomers.

For a closer study of the cyclization reaction it is essential to know the steric configuration of the diastereomers of the α,α'-dibromodicarboxylic acids in question. For the α,α'-dibromoadipic acid, conclusive evidence is available (4) indicating that the high melting isomer has the meso configuration, but for α,α'-dibromopimelic acid a complete investigation of both the diastereomers is still missing. Fehnel and Oppenlander have, in their publication (1), given an exhaustive report of what is currently known about this pimelic acid derivative, concluding that the literature does not provide any information on the stereoisomeric forms. The authors (1) reinvestigated the preparation of the dibromo acid, slightly altering Willstätter's original procedure (5), and succeeded in isolating a product, m.p. 155°, to which they ascribed the meso configuration. The low melting isomer could, however, not be characterized, but it was observed that the mother liquors (formic acid solution) slowly deposited additional quantities of the above mentioned acid. This was explained by the authors as due to gradual conversion of the more soluble form into the less soluble *via* inversion at one of the two asymmetric centres. Such an inversion is well known for halogen derivatives. It is caused by exchange with halogen ions in solution, and has been kinetically studied in racemization processes of optically active compounds.—As has previously been mentioned (6) the present author also has investigated the bromination of pimelic acid. As that work is not yet finished, it is considered unnecessary to publish any experimental details, but some aspects may be pointed out. The author has independently obtained the same results for the high melting isomer of α,α′-dibromopimelic acid as those described by FEHNEL and OPPENLANDER. His experiences, however, show that the choice of solvent in the purification is important, as in experiments with solvents having considerably lower dielectric constants than that of formic acid, mainly toluene, difficulties were encountered in obtaining the stereochemically pure meso form. In spite of numerous recrystallizations from toluene the melting point did not rise above 146–147°. In resolution experiments on this acid with brucine in acetone-water solution the primary salt yielded an optically active acid (dextrorotatory), thus indicating the presence of the racemic form. The pure high melting form (m.p. 155°) gives no resolution under analogous conditions. Although the solubility conditions in formic acid seem suitable for a separation of the diastereomers, it must be emphasized that the conversion mentioned by FEHNEL and OPPENLANDER, the rate of which varies with the polarity of the solvent, is important and probably responsible for the differences observed. Certain difficulties in separating the diastereomers could possibly be expected, as the differences in properties decrease with increasing length of the carbon chain. This can be seen for α,α′-dimethylpimelic acid (7), where simple fractional crystallization does not yield the pure isomers.

FEHNEL and OPPENLANDER ascribed the less soluble form of α,α′-dibromopimelic acid the meso configuration, as the cyclization reaction with sodium sulphide yielded *cis*-2,6-tetrahydrothiapyrandicarboxylic acid. The cis structure of this acid is obvious from the fact that dehydration in boiling acetic anhydride gives a cyclic anhydride, from which the original acid can be regenerated by the addition of water. The cyclic anhydride can have only the cis configuration (molecular model studies), and no rearrangement is to be expected under the conditions employed for the hydrolysis of the anhydride to the dicarboxylic acid.

According to the previous discussion a treatment of the mixture of the diastereomers of α,α′-dibromopimelic acid with aqueous sodium sulphide should yield the cis and trans isomers of the cyclic sulphide dicarboxylic acid. Starting from an α,α′-dibromopimelic acid, m.p. 136–144°, a crude product was obtained, which on recrystallization from water gave the pure cis form. The mother liquor was evaporated, leaving a residue with a composition approximately corresponding to that calculated for tetrahydrothiapyrandicarboxylic acid. The solubility conditions for the acids did not allow a separation by fractional crystallization, but a method involving the calcium salts was found suitable. A concentrated ammoniacal solution of the crude product was thus treated on a water bath with a saturated solution of calcium chloride. The calcium salt of the cis form, which separated, was filtered off, and from the filtrate a low melting isomer could be isolated. Recrystallization from small amounts of water yielded an acid with the melting point 148–149°, isomer of the above mentioned cis form, m.p. 210–211°.—A similar method has been described for the separation of the cis and trans isomers of 1,3-cyclohexanedicarboxylic acid (8). In this case the difference in solubility is even more pronounced.

The racemic configuration of the low melting 2,6-tetrahydrothiapyrandicarboxylic acid could be established by preliminary resolution experiments. The acid strychnine salt in alcoholic solution gives the (−)-form, whereas the primary brucine salt in acetone-water yields the (+)-form. The high melting isomer gives with the same alkaloids salts, from which the inactive acid is recovered with unchanged melting point.—Further information from the stereochemical point of view can be obtained from the infrared spectra (Figs. 1 and 2). A distinct difference, particularly obvious in the longer wavelength region, shows that the low melting trans form is completely free from the cis isomer. It is further interesting to note a shift in the main carboxylic frequency of the cis form to longer wavelength, which is best interpreted as indicating a clear difference in the spatial distribution of the carboxylic groups of the isomers. The cis form has its carboxylic groups situated nearest to each other, which is also obvious from molecular model studies. A comparison with the behaviour of the diastereomers of α,α'-dimethylsubstituted aliphatic dicarboxylic acids is of interest (9 and 10). The infrared absorption curve for the unsubstituted thiacyclohexane has previously been reported (11 and 12).

Since the cyclic anhydride related to 2,6-tetrahydrothiapyrandicarboxylic acid can have only the cis configuration, it would be interesting to investigate the action of dehydrating agents on the trans acid. Refluxing with propionic anhydride was thus found to give the anhydride of the cis form, whereas acetyl chloride reacts only at higher temperatures under pressure, otherwise leaving the trans isomer unchanged.

For a complete study of the stereoisomers it should be valuable if the cis form could be rearranged to give the trans isomer. A partial conversion of that kind is possible for the analogous 1,3-cyclohexanedicarboxylic acid by heating with concentrated hydrochloric acid to 180° (8) or better by heating of the cis form at 170–180° for 20 hours (13). FREDGA has successfully used this sort of racemization process for the conversion of *cis*-1,2-dithiane-3,6-dicarboxylic acid into the corresponding trans isomer (14). A similar rearrangement is possible for 1,4-cyclohexanedicarboxylic acid, but the conversion proceeds much more easily for the disulphide acid. An analogous effect was now found for 2,6-tetrahydrothiapyrandicarboxylic acid, where the cis form could be rearranged by melting and heating at 215–220° for 12 minutes. The extent of conversion here was 58 %, whereas the corresponding value for the cyclohexane derivative was 30 %. The separation of the diastereomers was performed by means of the calcium salts.

It is of some interest to compare the thiacyclohexane derivatives in question with the corresponding cyclohexane compounds. The presence of a sulphur atom close to asymmetric centres usually facilitates steric conversions in general.—For the methyl or ethyl esters of 1,2-cyclohexanedicarboxylic acid HÜCKEL and GOTH have found an interesting rearrangement reaction (15). The action of small amounts of *e.g.* sodium ethylate causes a complete conversion of the cis ester to the trans isomer. The reaction has been explained by the dissociation of a proton from the asymmetric centre with plane configuration around that carbon atom as a consequence. The ester enolate then yields the trans isomer as it is more energetically stabilized. The same conversion has been reported for the dimethyl ester of *cis*-3,4-tetrahydrothiapyrandicarboxylic acid (16). This reaction seems very valuable for the synthesis of the trans isomers and has now been investigated for 2,6-tetrahydrothiapyrandicarboxylic acid. Experiments have been performed with a mixture of the stereoisomeric ethyl esters (obtained from a mixture of *racem* and *meso* ethyl ester of α,α'-dibromopimelic acid in reaction with potassium sulphide) and with both the stereochemically pure dimethyl esters (obtained from the corresponding acids and diazomethane). In this case hydrolysis only yielded a mixture

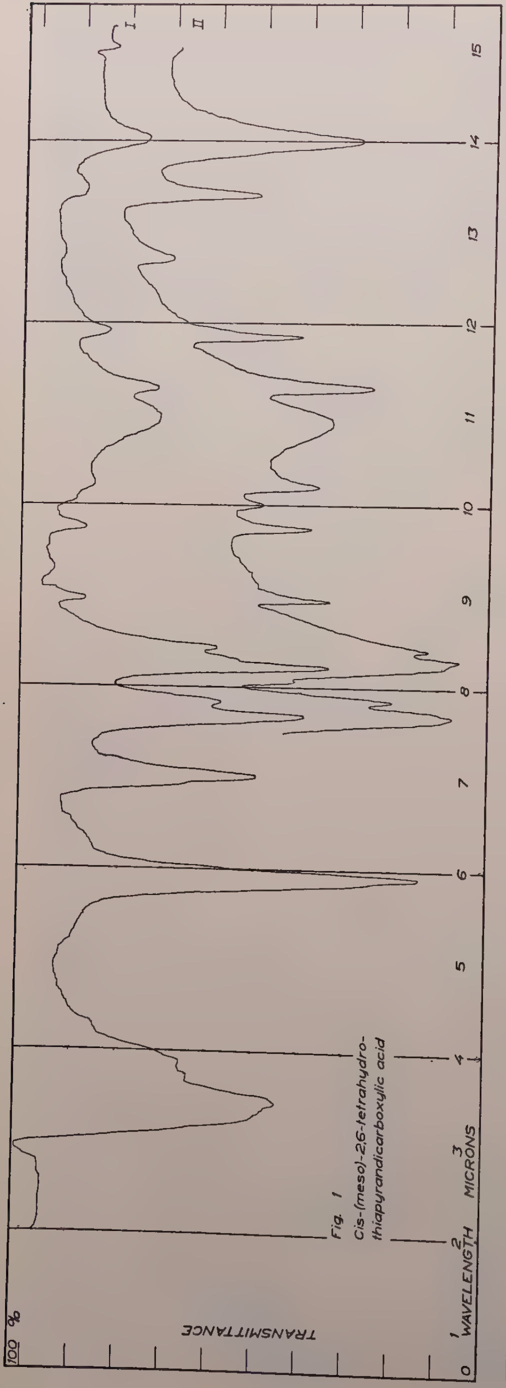


Fig. 1
Cis-(meso)-2,6-tetrahydrothiapyrandicarboxylic acid

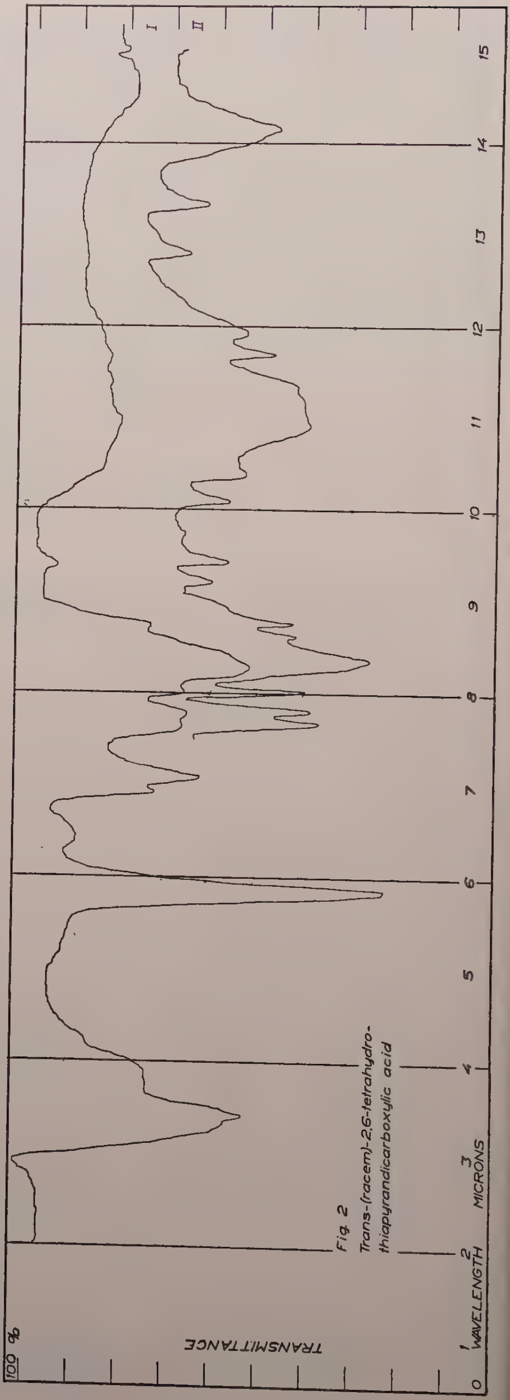


Fig. 2
Trans-(racem)-2,6-tetrahydrothiapyrandicarboxylic acid

of the diastereomers. It is thus obvious that the 1,2-position of the carboxylic groups favours the conversion as compared with the 1,3-position. It is of importance to note that the analogous 1,3-cyclohexanedicarboxylic acid exhibits very small differences in the heats of combustion for the cis and trans dimethylester. This shows that the trans isomer is stabilized only to a small degree. The special character of the 1,3-substituted cyclohexanes of the hydroaromatic series from the structural point of view (13 and 17) is surely important, and it seems justifiable to draw analogous conclusions for the cyclic sulphide dicarboxylic acid. That explains the failure of complete conversion as due to the small difference in structural stability for the diastereomers.—From the foregoing it is evident that a complete investigation of the heats of combustion for the diastereomers in question would be valuable.—It is hoped to pursue this investigation further.

Experimental

Cis-2,6-tetrahydrothiapyrandicarboxylic acid

This acid was prepared in the same manner as described by FEHNEL and OPPENLANDER (1). Recrystallization from glacial acetic acid or better from water. M.p. 210–211°.

Anal. Subst., 33.90 mg: 7.40 ml of 0.0482-N NaOH. Equ. wt. Calc. 95.1; found 95.0.

Trans-2,6-tetrahydrothiapyrandicarboxylic acid

A. Preparation from the diastereomer mixture of α,α'-dibromopimelic acid. 39.2 g (0.123 mole) of a mixture of *racem* and *meso* α,α'-dibromopimelic acid (equ. wt. 159.6 (calc. 159.0); m.p. 136–144°) was treated with sodium sulphide as described for the cis form. The solid residue after the ether evaporation was recrystallized directly from 20 ml of water to give 9.5 g of the cis isomer, m.p. 205–207°. The mother liquor was evaporated to dryness, and the crude product obtained, about 10 g, was dissolved in 30 ml of concentrated ammonia. A solution of 20 g of calcium chloride hexahydrate in 70 ml of water was added, and the mixture was stirred on a boiling water bath for about one hour. The less soluble calcium salt of the cis form separated gradually and was filtered off. An additional quantity of 2.2 g of cis form, m.p. 208°, was thus obtained on acidification. The filtrate after the separation of the cis isomer was acidified with hydrochloric acid and the acid solution extracted repeatedly with ether. On evaporation of the ether a colourless crystalline substance remained, m.p. 135–140°, which after recrystallization (3 times) from small amounts of water (or formic acid) yielded 3.0 g of *trans*-2,6-tetrahydrothiapyrandicarboxylic acid with the m.p. 148–149°.—A definite confirmation of the melting point may be obtained if the enantiomorphs are available.—It was sometimes necessary to repeat the separation procedure in order to get satisfactory results.—The yield was: 50 % of cis form and 13.4 % of trans form.

Anal. (of the trans form). Subst., 40.00 mg: 8.72 ml of 0.0482-N NaOH. Equ. wt. calc. 95.1; found 95.2.

B. Preparation from cis-2,6-tetrahydrothiapyrandicarboxylic acid. 15.0 g (0.079 moles) of *cis*-2,6-tetrahydrothiapyrandicarboxylic acid was placed in a large test-tube and immersed in an oil bath at 215–220°. The melting was hastened by stirring, and when the acid had melted completely (about 5 minutes), the heating was continued for 7 minutes. A slight evolution of gas was observed, and the mixture was somewhat discoloured. After cooling the product was treated with 10 ml of hot water, from which separated 13 g of an acid, m.p. 135–173°. This crude product was recrystallized from 23 ml of water to which was added some decolourising charcoal. 2 g of pure cis form, m.p. 205–207°, deposited at room temperature. The combined mother liquors were evaporated and the residue treated with calcium chloride in ammoniacal solution as above (A). In this manner 6.5 g of the trans isomer, m.p. 146–147° after two recrystallizations, and 2.7 g of the cis form, m.p. 207° after one recrystallization, was isolated. A mixed melting point showed no depression.—The result of the rearrangement reaction may thus be summarized as follows: 31 % of *cis*-2,6-tetrahydrothiapyrandicarboxylic acid was recovered, 43 % of the trans isomer was obtained, and 26 % was lost by decomposition or in some other way.—If the reaction time is made shorter a considerable quantity of the cis form remains unchanged. Heating over a longer period, on the other hand, causes too much decomposition.

Anal. (of the trans form). Subst., 30.62 mg: 9.61 ml of 0.0334-N NaOH. Equ. wt. Calc. 95.1; found 95.4.

Preliminary experiments on resolution

(+)-*Form.* 0.19 g (0.001 mole) of *trans*-2,6-tetrahydrothiapyrandicarboxylic acid and 0.47 g (0.001 mole) of brucine were dissolved in a hot mixture of acetone (5 ml) and water (1 ml). Crystallization over-night at room temperature yielded 0.35 g (53 %) of salt, from which the acid was liberated in the usual manner. The rotatory power was measured in alcohol. $[\alpha]_D^{25} = +14.3°$.

Anal. Subst., 15.41 mg: 7.72 ml of 0.0211-N NaOH. Equ. wt. Calc. 95.1; found 94.6.

(−)-*Form.* 0.19 g (0.001 mole) of the trans acid and 0.33 g (0.001 mole) of strychnine yielded in 10 ml of alcohol 0.24 g (46 %) of salt. The rotatory power was measured as described above. $[\alpha]_D^{25} = -62.3°$.

Anal. Subst., 16.20 mg: 7.98 ml of 0.0211-N NaOH. Equ. wt. Calc. 95.1; found 96.2.

From the neutral brucine and strychnine salts of *trans*-2,6-tetrahydrothiapyrandicarboxylic acid acids were obtained which showed little or no activity.—The acid and neutral brucine salts of *cis*-2,6-tetrahydrothiapyrandicarboxylic acid, crystallized from acetone-water under conditions similar to those described above, gave inactive acids on regeneration with unchanged melting point, 210°. The same was valid for the primary strychnine salt.

Cis-2,6-tetrahydrothiapyrandicarboxylic anhydride

A. Preparation from cis-2,6-tetrahydrothiapyrandicarboxylic acid. 1 g of the acid was refluxed with 8 ml of propionic anhydride for two hours. The product was isolated in the same manner as described by Fehnel and Oppenlander (1). M.p. 168°. The cis acid was recovered by treatment of the anhydride with water.

B. Preparation from trans-2,6-tetrahydrothiapyrandicarboxylic acid. 1 g of the acid was treated with 8 ml of propionic anhydride as described above. The crude product was directly characterized without isolation by dissolving in hot water (3 ml) and the solution upon cooling deposited 0.6 g (60 %) of the *cis acid*, m.p. 208°.

Treatment of 1 g of *trans*-2,6-tetrahydrothiapyrandicarboxylic acid with 6 ml of acetyl chloride under reflux for 3 hours did not cause anhydride formation to any mentionable extent. 0.85 g (85 %) of the trans acid was recovered on hydrolysis, m.p. 145–146°.—A partial conversion was observed, when the reaction was performed in a closed tube at 150°. After hydrolysis 0.3 g (30 %) of the cis acid, m.p. 208–209°, was isolated by means of the calcium salt.

Rearrangement experiments on esters of 2,6-tetrahydrothiapyrandicarboxylic acid

Diethyl 2,6-tetrahydrothiapyrandicarboxylate was prepared from ethyl α,α'-dibromopimelate, b.p. 138–148° at 0.6–1 mm., and potassium sulphide in alcohol solution. The method is analogous to that used by FEHNEL and OPPENLANDER (1) for the methyl ester. 15 g (0.06 moles) of this ethyl ester, b.p. 125–130° at 1 mm., was treated for half an hour on a water bath with 0.14 g of sodium in 5 ml of alcohol. Hydrolysis with sodium hydroxide in ethanol then gave after acidification a mixture of the diastereomeric acids of which only the cis form was isolated in a pure state. 6 g (53 %), m.p. 207–208°, was obtained, and no melting point depression was found when mixed with cis acid, prepared as described above.

Cis and trans dimethyl 2,6-tetrahydrothiapyrandicarboxylate were prepared from the corresponding pure acids, m.p. 210–211° and 148–149° respectively, and diazomethane. 1 g of each ester, of which the cis isomer was solid, m.p. 40–43°, was treated with 25 mg of sodium in 5 ml of methanol as described above. The acids obtained after hydrolysis melted over a wide range of temperature indicating conversion. Approximately equal amounts of each diastereomer were found after separation by means of the calcium salts.

Infrared absorption measurements

A PERKIN ELMER model 21 spectrophotometer equipped with sodium chloride prisms was used for the infrared absorption measurements. The samples were examined as solid pressed potassium bromide plates (curve I in the figures) and as nujol mulls (curve II in the figures) having 6–7 times higher concentration. The absorption curves I (Figs. 1 and 2) are discontinuous at 7.24 μ due to a sharp absorption band caused, according to MECKE (cf. 10), by nitrogen impurities in the potassium bromide.

ACKNOWLEDGEMENTS

The author is indebted to Professor ARNE FREDGA for valuable discussions. The author also wishes to express his gratitude to Dr. ANDREAS ROSENBERG for the infrared work of this investigation and for his expert advice.

A grant from the *Swedish Natural Science Research Council* is gratefully acknowledged.

SUMMARY

A contribution to the knowledge of the stereoisomers of α,α'-dibromopimelic acid is given.

Both the diastereomers of 2,6-tetrahydrothiapyrandicarboxylic acid have been prepared and characterized.

Some rearrangement reactions have been investigated and the results compared with what is known for the analogous cyclohexane series.

Infrared spectra of the thiapyran acids are given.

Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala, May 1954.

REFERENCES

1. E. A. Fehnel and G. C. Oppenlander, J. Am. Chem. Soc. *75,* 4660 (1953).
2. A. Fredga, J. prakt. Chem. *150,* 124 (1938).
3. L. Schotte, Acta Chem. Scand. *8,* 130 (1954).
4. B. Holmberg and E. Müller, Ber. *58,* 1601 (1925).
5. R. Willstätter, Ber. *28,* 655 (1895).
6. L. Schotte, Acta Chem. Scand. *8,* 131 (1954).
7. F. S. Kipping, J. Chem. Soc. *67,* 139 (1895).
8. W. Goodwin and W. H. Perkin, J. Chem. Soc. *87,* 841 (1905).
9. A. Rosenberg and L. Schotte, Acta Chem. Scand. *8,* 867 (1954).
10. L. Schotte and A. Rosenberg, Arkiv Kemi *7,* 479 (1954).
11. A. Müller, E. Funder-Fritzsche, W. Konar and E. Rintersbacher-Wlasak, Monatsh. *84,* 1206 (1953).
12. N. J. Leonard and J. Figueras, J. Am. Chem. Soc. *74,* 918 (1952).
13. A. Skita and R. Rössler, Ber. *72,* 265 (1939).
14. A. Fredga, Arkiv Kemi, Mineral. Geol. *12 A* (1938) no. 27.
15. W. Hückel and E. Goth, Ber. *58,* 447 (1925).
16. B. R. Baker and F. Ablondi, J. Org. Chem. *12,* 328 (1947).
17. H. A. Smith and F. P. Byrne, J. Am. Chem. Soc. *72,* 4406 (1950).

Tryckt den 18 november 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 53

Vorgelegt am 2. Juni 1954

Zur Kenntnis der Zuckerbenzhydrazide

Von **Bror Holmberg**

1. Übersicht

Zwecks Vergleiches mit den durch ein vielseitiges Reaktionsvermögen ausgezeichneten Zuckerthiobenzhydraziden (1) wurden die entsprechenden, allerdings in mehreren Fällen schon bekannten, schwefelfreien Zuckerbenzhydrazide etwas näher studiert. Eine Übersicht über die Schmelzpunkte und Drehungsvermögen der hierbei[1] untersuchten Verbindungen zeigt die folgende Tabelle.

Benzhydrazid der	Schmelzpunkt[2]	$[\alpha]_D^{20}$ [3]
d (−)-Arabinose	184°–186°; 196°–198°[4]	$\xrightarrow{300\text{ Min.}} -25{,}3^\circ \longrightarrow x$[5]
l (+)-Arabinose	184°–186°; 196°–198°[6]	$\xrightarrow{10} +16{,}8^\circ \xrightarrow{240} +25{,}8^\circ \longrightarrow \xrightarrow{5040} +20{,}9^\circ \longrightarrow x$
d, l-Arabinose	176°–178°; 187°–189°	—
d (+)-Xylose	164°–166°; 166°–168°[7]	$+14{,}4^\circ \xrightarrow{300} +10{,}2^\circ \xrightarrow{1440} \longrightarrow +5{,}9^\circ \longrightarrow x$
d (−)-Lyxose[8]	147°–150°; 150°–154°	$-8{,}6^\circ$[9]
l (+)-Rhamnose	175°–177°; 186°–188°[10]	$+21{,}1^\circ \xrightarrow{2880} +11{,}1^\circ$
l (−)-Fucose	177°–179°; 189°–190°[11]	$-6{,}4^\circ \xrightarrow{720} -17{,}6^\circ$
d (+)-Glucose	177°–179°; 186°–188°[12]	$-5{,}1^\circ \xrightarrow{1440} +0{,}7^\circ \xrightarrow{5760} -4{,}1^\circ$
d (+)-Mannose	177°–179°; 183°–184°[13]	$-27{,}4^\circ \xrightarrow{720} -14{,}9^\circ$
d (+)-Galaktose	174°–176°; 184°–186°[14]	$+1{,}9^\circ \xrightarrow{420} +13{,}1^\circ \xrightarrow{2880} +10{,}5^\circ$
Lactose[15]	178°–180°; 182°–184°	$-1{,}0^\circ \xrightarrow{720} +5{,}9^\circ$[9]

---

[1] Von der *d*-Ribose, der *d*-Fruktose, der Maltose und der Cellobiose konnten keine kristallisierten Benzhydrazide erhalten werden.

[2] Das Schmelzen findet stets unter Braunfärbung und Gasentwicklung statt; die Schmelzpunkte sind daher stark von der Erhitzungsgeschwindigkeit abhängig. Um eine Vorstellung hierüber zu geben, sind in der Tabelle sowohl die bei langsamen (erste Angaben) wie die bei schnellen (zweite Angaben) Erhitzungen gefundenen Schmelz- oder Zersetzungstemperaturen angeführt.

[3] In 50 % überschüssiger Natronlauge und für c = ca. 4 g (bei der Lactose 6 g) in 100 ml.

[4] Hirst und Mitarbeiter (2) geben 186° an.

[5] Wegen Verfärbung konnte kein Endwert bestimmt werden.

[6] Subaschow (3) fand 184°, Davidis (4) 211°–212°, van der Haar (5) 212° und Hirst und Mitarbeiter (2) 186°–190°.

[7] van der Haar (5) fand 176°.

[8] Mit einem Mol. Kristallwasser.

[9] Für wasserfreie Sbst. berechnet.

[10] Hirst und Mitarbeiter (2) fanden 180°, van der Haar (5) 189°.

[11] van der Haar (5) fand 197°.

[12] Wolff (6) fand 171°–172°, Lobry de Bruyn und Alberda v. Ekenstein (7) 186°–187°, Davidis (4) 195°–196° und van der Haar (5) 171°–172°.

[13] van der Haar (5) fand 185°.

[14] Gemäss Schubaschow (3) sowie van der Haar (5) 178°.

[15] Mit 3 Mol. Kristallwasser.

Wie schon die ersten Darsteller[1] von Zuckerbenzhydraziden fanden, können diese Verbindungen beim Erhitzen mit Wasser hydrolytisch in ihre Komponenten gespalten werden; aus den unten bei den Arabinose- und Glucosebenzhydraziden beschriebenen Versuchen geht hervor, dass diese Spaltung von Salzsäure stark beschleunigt wird. Als Folge dieser Unbeständigkeit wurden bei den Umsetzungen dieser Benzhydrazide mit Eisenchlorid und mit Formaldehyd nur Derivate des Benzhydrazids selber erhalten, während bei der Einwirkung von Thiobenzhydrazid auf das Glucosebenzhydrazid erst nach dem Zusatz von Salzsäure Reaktion eintrat, wobei das Thiobenzhydrazid der Glucose entstand.

Diese Untersuchung wurde in der damaligen org.-chem. Abteilung des Nobelinstitutes der Kgl. Akademie der Wissenschaften, Stockholm, ausgeführt. Das benutzte Benzhydrazid wurde durch Umsatz der Benzoylthioglykolsäure mit Hydrazin (8) dargestellt; die Analysen verdankt der Verfasser Frau MAJBRITT VESTERGAARD.

2. Arabinosebenzhydrazide

Beim Erhitzen auf dem Wasserbade von 3,0 g *l*-Arabinose in 25 ml Wasser mit 2,8 g Benzhydrazid begann bald die entstandene Lösung Kriställchen abzuscheiden; nach einer Stunde und Erkalten wurden 4,8 g farblose, schmale Täfelchen oder dünne Prismen erhalten, welche unter Zersetzung bei 186°–188° bzw. 192°–193° schmolzen.

Für $C_{12}H_{16}O_5N_2$ (268,26):

ber.: C 53,72, H 6,01, N 10,44 %,
gef.: » 53,51, » 6,00, » 10,51 ».

Eine Lösung von 0,4115 g Sbst. mit 2,30 ml 1-n. Natronlauge und Wasser auf 10,00 ml Volumen zeigte *t* Minuten nach dem Zusatz der Lauge:

t:	10	15	20	30	60	120	240	480
α_D^{20}:	+ 0,69°	+ 0,75°	+ 0,80°	+ 0,88°	+ 0,98°	+ 1,05°	+ 1,06°	+ 1,03°
	720	1440	2160	3600	5040			
	+ 1,00°	+ 0,96°	+ 0,95°	+ 0,93°	+ 0,86°,			

Lösung dann bräunlich gelb.

$$[\alpha]_D^{20} = \xrightarrow{10} + 16{,}8° \xrightarrow{240} + 25{,}8° \xrightarrow{5040} + 20{,}9° \xrightarrow{\infty} x$$

$$[M]_D^{20} = \xrightarrow{10} + 45{,}0° \xrightarrow{240} + 69{,}1° \xrightarrow{5040} + 56{,}1° \xrightarrow{\infty} x.$$

Als eine Lösung von 3,0 g *l*-Arabinose in 10 ml Wasser mit 2,8 g Benzhydrazid in 10 ml heissem Alkohol versetzt wurde, entstand ein homogenes Gemisch, welches nach 3/4 Stunden sich zu trüben begann und am folgenden Tage 5,0 g mit 5 ml 50%igem Alkohol gewaschenes Kristallpulver vom Schmp. 184°–186° bzw. 193°–195° lieferte. Dieses Produkt enthielt 53,64 % C, 6,06 % H und 10,52 % N; in Natronlauge wie oben zeigte es

$$[\alpha]_D^{20} = \xrightarrow{10} + 16{,}7° \xrightarrow{240} + 25{,}2° \xrightarrow{5040} + 20{,}3°.$$

In der gleichen Weise lieferten 3,0 g *l*-Arabinose in 10 ml 2-n. Salzsäure mit 2,8 g Benzhydrazid in 10 ml heissem Alkohol usw. 2,6 g *l*-Arabinosebenzhydrazid vom Schmp. wie oben und

$$[\alpha]_D^{20} = \xrightarrow{10} + 16{,}3° \xrightarrow{240} + 25{,}5° \xrightarrow{5040} + 20{,}6°.$$

---

[1] SUBASCHOW (3) und WOLFF (6).

Die Mutterlauge wurde mit einer Lösung von 3 g Natriumazetat in 5 ml Wasser versetzt und ging dann schnell in einen Brei aus 2,1 g Kristallschuppen über, welche wie das vorige Produkt schmolzen und wie oben

$$[\alpha]_D^{20} = \xrightarrow{10} +16,7^\circ \xrightarrow{240} +26,1^\circ \xrightarrow{5040} +21,0^\circ$$

zeigten.

Beim Umkristallisieren von 2,0 g rohen *l*-Arabinosebenzhydrazids aus 100 ml Wasser wurden 0,7 g glänzende, schmale Schuppen erhalten, welche bei 184°–186° bzw. 196°–198° schmolzen und in Natronlauge wie oben nach 300 Minuten $[\alpha]_D^{20} = +25,6^\circ$ zeigten.

Eine in der Wärme bereitete Lösung von 0,2541 g *l*-Arabinosebenzhydrazid in Wasser auf 20,04 ml Volumen zeigte sogleich nach der Temperierung sowie 4 Stunden später $2\,\alpha_D^{20} = +1,11^\circ$, $[\alpha]_D^{20} = +43,8^\circ$ und $[M]_D^{20} = +117,4^\circ$.

Beim Schütteln von 0,4062 g *l*-Arabinosebenzhydrazid mit 4,0 ml 1-n. Salzsäure entstand in 30 Minuten eine klare Lösung, welche nach Verdünnen mit Wasser auf 20,04 ml Volumen nach 10, 60 und 600 Minuten nach dem Zusatz des Wassers $2\,\alpha_D^{20} = +2,37^\circ$, $[\alpha]_D^{20} = +58,5^\circ$ und $[M]_D^{20} = +156,8^\circ$ zeigte. Eine Lösung von 0,2273 g *l*-Arabinose in 4,0 ml 1-n. Salzsäure und Wasser wie oben zeigte andererseits nach *t* Minuten:

t:	5	10	15	35	65
$2\,\alpha_D^{20}$:	+ 2,41°	+ 2,23°	+ 2,35°	+ 2,35°	+ 2,35,°

woraus als Endwerte $[\alpha]_D^{20} = +103,6^\circ$ und $[M]_D^{20} = +155,5^\circ$ erhalten werden; in der salzsauren Lösung dürfte daher das Arabinosebenzhydrazid vollständig in seine Komponenten gespalten worden sein.

Aus 6 g *d*-Arabinose in 20 ml Wasser und 5,4 g Benzhydrazid in demselben Volumen Alkohol wurden wie bei der *l*-Arabinose oben 9 g Umsetzungsprodukt vom Schmp. 187°–189° bzw. 191°–192° und in Natronlauge wie gewöhnlich nach 300 Minuten von $[\alpha]_D^{20} = -25,5^\circ$ erhalten. Beim Umkristallisieren von 2,0 g davon aus 50 ml 50 %-igem Alkohol wurden 1,55 g glänzende Schuppen vom Schmp. 184°–186° bzw. 196°–198° und in Natronlauge wie oben nach 300 Minuten von $[\alpha]_D^{20} = -25,3^\circ$ erhalten.

Als ein Gemisch aus je 1,0 g der beiden aktiven Formen des Arabinosebenzhydrazids aus 100 ml Wasser umkristallisiert wurde, schied die Lösung beim Erkalten und bis zum folgenden Tage 0,15 g der inaktiven Form als schmale Täfelchen oder flache Prismen vom Schmp. 176°–178° bzw. 187°–189° aus. Nach Eindunstung der Mutterlauge auf dem Wasserbade zu 50 g Gewicht wurden dann noch 0,55 g derselben Form vom Schmp. wie vorher und mit 10,40, ber. 10,44, % N gewonnen.

Beim Schütteln eine halbe Stunde von 2,7 g des *l*-Arabinosebenzhydrazids mit 25 ml 1-n. Natronlauge wurden 2,4 g davon gelöst; diese Lösung lieferte mit 2 g *Formalin* ein homogenes Gemisch, welches bis zum folgenden Tage 0,25 g schwach cremefarbenes Pulver vom Schmp. ca. 135°–145° (Präp. 1) ausschied. Das Filtrat davon wurde mit 10 ml 2-n. Essigsäure versetzt und ging dabei in eine Emulsion über, welche bis zum folgenden Tage 0,7 g weisses Pulver vom Schmp. ca. 170°–190° (Zersetzung) und der schwer zu deutenden Zusammensetzung $C_{11}H_{14}O_2N_4$ absetzte. Die Mutterlauge schied bei der freiwilligen Eindunstung auf 7 g Gewicht noch 0,1 g Pulver aus; das Filtrat davon lieferte mit 1,5 g

Thiobenzhydrazid in 10 ml Alkohol 1,2 g *l*-Arabinosethiobenzhydrazid vom Schmp. 165°–167°, mit 11,19, ber. 11,28, % S und in Pyridin von $[\alpha]_D^{20} = -36{,}8^\circ$.

Eine heisse Lösung von 2,7 g *l*-Arabinosebenzhydrazid in 100 ml 50%igem Alkohol, welche beim Erkalten bei ca. 50° zu kristallisieren begann, lieferte dann mit 2 g Formalin ein homogenes Gemisch, welches beim Eindunsten auf 15 g Gewicht 1,4 g schmale Prismen vom Schmp. ca. 167°–169° (Präp. 2) ausschied. Aus der Mutterlauge wurden hier wie oben 2,2 g *l*-Arabinosethiobenzhydrazid vom Schmp. 167°–168° und $[\alpha)_D^{20} = -37{,}2^\circ$ gewonnen. — Als 2,7 g *l*-Arabinosebenzhydrazid mit 1,0 g Formalin in 20 ml 1-n. Salzsäure geschüttelt wurden, trat keine sichtbare Auflösung ein, aber nach 12 Stunden wurden beim Absaugen 1,6 g weisses Pulver vom Schmp. ca. 140°–170° erhalten (Präp. 3); das Filtrat ergab wie oben 2,1 g *l*-Arabinosethiobenzhydrazid vom Schmp. 167°–168° und $[\alpha]_D^{20} = -37{,}1^\circ$. — Bei einstündigem Schütteln von 2,7 g *l*-Arabinosebenzhydrazid mit 20 ml 1-n. Salzsäure entstand eine Lösung, welche beim Zusatz von 2 g Formalin sogleich ein aus dichten Bällen von kleinen Prismen bestehendes, farbloses Pulver von 1,5 g Gewicht und Schmp. ca. 143°–145° (–155°) ausschied (Präp. 4). — Als zum Schluss eine Lösung von 1,4 g des Benzhydrazids selber in 20 ml gew. Alkohol mit 1,0 g Formalin versetzt wurde, trat nach ca. 10 Minuten Trübung ein; bis zum folgenden Tage wurden 1,1 g Kristallpulver vom Schmp. ca. 169°–171° ausgeschieden (Präp. 5).

Die Analysen der verschiedenen Präparate ergaben die ungefähren Zusammensetzungen einer gemäss der Gleichung

$$2\,C_7H_8ON_2 + 2\,CH_2O = C_{16}H_{18}O_3N_4 + H_2O$$

entstandenen Verbindung:

Für $C_{16}H_{18}O_3N_4$ (314,34):

	ber.:		C 61,13,	H 5,77,	N 17,83 %,
gef.,	Präp.	1:	» 60,84,	» 5,77,	» 18,45 »,
»	»	2:	» 62,06,	» 5,53,	» 17,96 »,
»	»	3:	» 61,33,	» 5,82,	» 17,83 »,
»	»	4:	» 61,26,	» 5,66,	» 17,84 »,
»	»	5:	» 62,00,	» 5,63,	» 17,89 ».

Als eine Lösung von 2,7 g *l*-Arabinosebenzhydrazid in 20 ml 1-n. Salzsäure mit 10 ml 2-m. *Eisenchlorid*-Lösung versetzt wurde, entstand ein homogenes Gemisch, welches bis zum folgenden Tage 0,05 g Kristallpulver vom Schmp. ca. 225°–228° ausschied. Die Mutterlauge wurde mit einer Lösung von 4 g Natriumazetat in 10 ml Wasser versetzt, wonach sie noch 0,6 g Pulver vom Schmp. ca. 220° absetzte. Beim Umkristallisieren aus 20 ml gew. Alkohol lieferte dieses Produkt 0,3 g des früher von STRUVE (9) durch Oxydation des Benzhydrazids selbst mit Jod erhaltenen sym.-Dibenzoylhydrazins vom Schmp. 237°–238° (gemäss STRUVE 233°).

Für $C_{14}H_{12}O_2N_2$ (240,25):

ber.:	C 69,98,	H 5,03,	N 11,67 %,
gef.:	» 69,76,	» 5,09,	» 11,72 ».

Eine Lösung von 2,7 g Arabinosebenzhydrazid in 20 ml 1-n. Natronlauge ging nach Zusatz von 20 ml 1-m. Eisenchlorid-Lösung unter etwas Gasentwicklung in einen Brei aus 0,9 g eines feinkörnigen, undeutlich kristallinischen Pulvers vom Schmp. ca. 215° über; als eine ähnliche Lösung mit 3,4 g *Pyridiniumtribromid* in 20 ml Alkohol versetzt wurde, traten schwache Wärme- und Gasentwicklungen ein und es wurden bis zum folgenden Tage 0,3 g farblose Nädelchen vom Schmp. 238°–240° ausgeschieden.

3. *d*-Xylosebenzhydrazid

Als eine Lösung von 3,0 g *d*-Xylose in 10 ml Wasser mit 2,8 g Benzhydrazid in 10 ml heissem Alkohol versetzt wurde, entstand ein homogenes Gemisch, welches sich bis zum folgenden Tage nicht veränderte, aber nach Zusatz einer bei freiwilliger Eindunstung kristallisierten Probe im Laufe einer Woche 2,5 g mit 15 ml 50%igem Alkohol gewaschenes Xylosebenzhydrazid als dicke Tafeln vom Schmp. 164°–166° bzw. 165°–167° (Zersetzung) absetzte.

Für $C_{12}H_{16}O_5N_2$ (268,26):

ber.:	**C 53,72,**	**H 6,01,**	**N 10,44 %,**
gef.:	**» 53,54,**	**» 5,94,**	**» 10,44 ».**

Von diesem Produkte zeigten 0,4134 g mit 2,31 ml 1-n. Natronlauge und Wasser zu 10,00 ml gelöst:

t:	0	10	30	70	300	720	1440	3000	5000
α_D^{20}:	+0,59°[1]	+0,58°	+0,57°	+0,55°	+0,41°	+0,32°	+0,25°	+0,12°	−0,05°

$$[\alpha]_D^{20} = +14,3^\circ \xrightarrow{300} +9,9^\circ \xrightarrow{1440} +6,0^\circ \xrightarrow{\infty} x.^2$$

Die Mutterlauge des obigen Produktes ergab bei der freiwilligen Eindunstung zu 5 g Gewicht 2,2 g einer mit 5 ml Wasser gewaschenen Kristallmasse vom Schmp. 161°–163° und wie oben von $[\alpha]_D^{20} = +14,1^\circ \xrightarrow{300} +10,1^\circ \xrightarrow{1440} +5,7^\circ$; bei der Umkristallisierung von 1,8 g davon aus 36 ml gew. Alkohol wurden 0,9 g farblose Tafeln oder flache Prismen erhalten, welche bei 164°–165,5° bzw. 166°–168° (Zers.) schmolzen und in Natronlauge wie oben

$$[\alpha]_D^{20} = +14,4^\circ \xrightarrow{300} +10,2^\circ \xrightarrow{1440} +5,9^\circ \xrightarrow{\infty} x,$$
$$[M]_D^{20} = +38,6^\circ \xrightarrow{300} +27,4^\circ \xrightarrow{1440} +15,8^\circ \xrightarrow{\infty} x,$$

zeigten.

Bei einstündiger Schüttelung von 1,8 g *d*-Xylosebenzhydrazid mit 25 ml Wasser gingen 0,95 g davon in Lösung; nach dem Zusatz von 2 g *Formalin* zu der Lösung lieferte das Gemisch im Laufe von 4 Tagen 0,45 g weisses Pulver vom Schmp. ca. 168°–170° (nach bei ca. 155° beginnender Sinterung) und der gleichen Zusammensetzung wie bei den entsprechenden Produkten aus dem Arabinosebenzhydrazid.

Für $C_{16}H_{18}O_3N_4$ (314,34):

ber.:	**C 61,13,**	**H 5,77,**	**N 17,83 %,**
gef.:	**» 60,93,**	**» 5,73,**	**» 17,87 ».**

---

[1] Geschätzt.

[2] Eintretende Verfärbung machte die Bestimmung eines Endwertes unmöglich.

4. *d*-Lyxosebenzhydrazid

Eine Lösung von 1,5 g *d*-Lyxose in 5 ml Wasser lieferte mit 1,4 g Benzhydrazid in 5 ml heissem Alkohol ein homogenes Gemisch, welches sich bis zum folgenden Tage nicht veränderte, aber nach dem Impfen mit einer kristallisierten Eindunstungsprobe im Laufe von 4 Tagen 1,1 g farblose, fast nadelförmige Prismen vom Schmp. 147°–150° bzw. 105°–154° (Zers.) ausshied. In zugeschmolzenem Röhrchen ging das Produkt bei ca. 115° in eine halbflüssige Masse über, welche 10 Grade höher eine klare Schmelze ergab.

Für $C_{12}H_{16}O_5N_2, 2\,H_2O = C_{12}H_{20}O_7N_2$ (304,30):

ber.:	C 47,36,	H 6,62,	N 9,21,	H_2O 11,84 %,
gef.:	» 47,48,	» 6,59,	» 9,25,	» 11,45 ».[1]

Aus der Mutterlauge wurden bei freiwilliger Eindunstung wie bei der Xylose noch 1,4 g Kristalle vom Schmp. wie oben erhalten.

Von dem zuerst gewonnenen Produkte zeigten 0,4215 g (= 0,3716 g wasserfreie Sbst.) mit 2,07 ml 1-n. Natronlauge und Wasser auf 10,00 ml Volumen gelöst nach 10, 20 und 240 Minuten nach dem Zusatz der Lauge $\alpha_D^{20} = -0{,}32°$, für wasserfreie Sbst. gef. $[\alpha]_D^{20} = -8{,}6°$ und $[M]_D^{20} = -23{,}1°$. Das gleiche Drehungsvermögen zeigte das Endprodukt.

5. *l*-Rhamnosebenzhydrazid

Beim Erhitzen auf dem Wasserbade von 1,8 g *l*-Rhamnose in 10 ml Wasser mit 1,4 g Benzhydrazid entstand sofort eine klare Lösung, welche nach 4 Stunden und Erkalten in einen Brei aus äusserst kleinen Prismen überging. Diese wurden am folgenden Tage abgesaugt, mit 5 ml Wasser gewaschen und auf Tonscherben getrocknet, wonach sie 2,4 g wogen und unter Zersetzung bei 175°–177° bzw. 185°–186° schmolzen.

Für $C_{13}H_{18}O_5N_2$ (282,29):

ber.:	C 55,31,	H 6,43,	N 9,92 %,
gef.:	» 55,20,	» 6,41,	» 9,97 ».

Eine Lösung von 0,4163 g des erhaltenen Rhamnosebenzhydrazids mit 2,21 ml 1-n. Natronlauge und Wasser auf 10,00 ml Volumen zeigte:

t:	0	10	20	60	240	720	1440	2880	4320
α_D^{20}:	+ 0,84°[2]	+ 0,83°	+ 0,82°	+ 0,78°	+ 0,62°	+ 0,52°	+ 0,46°	+ 0,46°	+ 0,46°.

$$[\alpha]_D^{20} = +20{,}2° \xrightarrow{1440} +11{,}0°.$$

Bei der freiwilligen Eindunstung der Mutterlauge des vorigen Produktes auf 3 g Gewicht wurden noch 0,3 g mit 3 ml Wasser gewaschenes Hydrazid vom Schmp. 180°–182° (Zers.) erhalten.

Als eine Lösung von 1,8 g *l*-Rhamnose in 5 ml Wasser mit 1,4 g Benzhydrazid in 5 ml heissem Alkohol versetzt wurde, entstand ein homogenes Gemisch, welches am folgenden Tage unverändet war, aber nach Impfen mit dem früher erhaltenen

---

[1] Gewichtsverlust im Vakuum über Phosphorsäureanhydrid.

[2] Geschätzt.

Produkte im Laufe von 3 Tagen 2,1 g mit 5 ml 50%igem Alkohol gewaschenes *l*-Rhamnosebenzhydrazid vom Schmp. 177°–179° bzw. 184°–185°, und wie oben von $[\alpha]_D^{20} = +21,0^\circ \rightarrow +11,9^\circ$ ausschied. Beim Umkristallisieren von 2,3 g eines Gemisches der erhaltenen Produkte aus 120 ml gew. Alkohol wurden 1,4 g haarfeine Nädelchen erhalten, welche bei 175°–177° bzw. 186°–188° schmolzen, 9,90, ber. 9,92, % N enthielten und in Natronlauge wie oben $[\alpha]_D^{20} = +21,1^\circ \xrightarrow{2880} +11,1^\circ$ und $[M]_D^{20} = +59,6^\circ \xrightarrow{2880} +31,3^\circ$ zeigten.

6. *l*-Fucosebenzhydrazid

Als eine Lösung von 1,2 g *l*-Fucose[1] in 10 ml Wasser mit 1,0 g Benzhydrazid auf dem Wasserbade erhitzt wurde, entstand eine klare Lösung, welche nach 4 Stunden unverändert war und dann beim Erkalten und bis zum folgenden Tage 1,5 g mit 5 ml Wasser gewaschene, farblose, schmale Täfelchen ausschied. Das so erhaltene *l*-Fucosebenzhydrazid schmolz unter Zersetzung bei 177°–179° bzw. 189°–190° und enthielt:

Für $C_{13}H_{18}O_5N_2$ (282,29):

ber.:	C 55,31,	H 6,43,	N 9,92 %,
gef.:	» 55,28,	» 6,41,	» 9,93 ».

Eine Lösung von 0,4368 g Sbst. mit 2,32 ml 1-n. Natronlauge und Wasser auf 10,00 ml Volumen zeigte:

t:	0	10	15	20	60	120	240	420
α_D^{20}:	− 0,28°[2]	− 0,30°	− 0,31°	− 0,33°	− 0,42°	− 0,53°	− 0,68°	− 0,75°
						720	1440	
						− 0,77°	− 0,77°	

$$[\alpha]_D^{20} = -6,4^\circ \xrightarrow{720} -17,6^\circ \text{ und } [M]_D^{20} = -18,1^\circ \xrightarrow{720} -49,8^\circ.$$

Die Mutterlauge des obigen Produktes schied bei der freiwilligen Eindunstung zu 3 g Gewicht noch 0,5 g Fucosebenzhydrazid vom Schmp. 175°–177° bzw. 185°–187° aus.

7. *d*-Glucosebenzhydrazid

Beim Vermischen einer Lösung aus 3,6 g *d*-Glucose in 10 ml Wasser mit 2,8 g Benzhydrazid in 10 ml heissem Alkohol entstand ein klares Gemisch, welches trotz Impfungen mit einer bei freiwilliger Eindunstung kristallisierten Probe erst nach einer Woche Kristalle abzuscheiden begann und dann im Laufe von 5 Tagen 2,9 g mit 5 ml 50%igem Alkohol gewaschene, farblose, schmale Prismen vom Schmp. 174°–176° bzw. 186°–188° (Zers.) ausschied. Aus der Mutterlauge wurden nach noch einer Woche 0,4 g Kristalle vom Schmp. 186°–187° (schnelles Erhitzen) und dann aus der neuen Mutterlauge beim Eindunsten auf 7 g Gewicht noch 1,8 g vom Schmp. 175°–177° bzw. 184°–186° gewonnen. Das zuerst ausgeschiedene Produkt ergab bei der Analyse:

---

[1] Bezogen von Pfanstiehl Chemical Co.

[2] Geschätzt.

Für $C_{13}H_{18}O_6N_2$ (298,29):

ber.:	C 52,34,	H 6,08,	N 9,39 %,
gef.:	» 52,25,	» 6,05,	» 9,38 ».

Das analysierte Produkt zeigte in Natronlauge wie unten

$$[\alpha]_D^{20} = -4{,}7^\circ \xrightarrow{720} +1{,}0^\circ \xrightarrow{5760} -3{,}8^\circ$$

und das zum Schluss erhaltene Produkt zeigte in der gleichen Weise

$$[\alpha]_D^{20} = -5{,}1^\circ \xrightarrow{720} +1{,}0^\circ \xrightarrow{5760} -3{,}8^\circ.$$

Aus 3,6 g *d*-Glucose, 2,8 g Benzhydrazid und 25 ml Wasser wurde auf dem Wasserbade eine klare Lösung erhalten, welche nach 4 Stunden sowie nach Erkalten und am folgenden Tage unverändert war, aber dann nach Impfung wie oben im Laufe einer Woche 2,7 g nadelförmige Prismen vom Schmp. 176°–178° bzw. 185°–187° absetzte. Dieses Produkt enthielt 52,34 % C, 6,04 % H und 9,39 % N und zeigte

$$[\alpha]_D^{20} = -4{,}6^\circ \xrightarrow{1440} +1{,}0^\circ \xrightarrow{5760} -3{,}9^\circ.$$

Die Mutterlauge ergab bei freiwilliger Eindunstung auf 9 g Gewicht noch 1,5 g Kristalle vom Schmp. 178°–180° bzw. 185°–187°, mit 52,27 % C, 6,04 % H und 9,48 % N und von

$$[\alpha]_D^{20} = -4{,}5^\circ \xrightarrow{1440} +1{,}0{,}^\circ \xrightarrow{5760} -3{,}6^\circ.$$

Beim Erhitzen während 5 Minuten von 3,0 g rohen *d*-Glucosebenzhydrazids mit 80 ml gew. Alkohol lösten sich 2,1 g davon; aus der Lösung wurden bis zum folgenden Tage 1,3 g Nadeln vom Schmp. 177°–179° bzw. 186°–188° ausgeschieden. Eine Lösung von 0,4110 g dieses Produktes in 2,07 ml 1-n. Natronlauge und Wasser auf 10,00 ml Volumen zeigte:

t:	0	10	20	60	120	240	420	720
α_D^{20}:	− 0,21°[1]	− 0,20°	− 0,19°	− 0,16°	− 0,12°	− 0,06°	− 0,03°	± 0,00°
			1440	2880	4320	5760	7080	
			+ 0,03°	− 0,09°	− 0,13°	− 0,17°	− 0,17°	

$$[\alpha]_D^{20} = -5{,}1^\circ \xrightarrow{1440} +0{,}7^\circ \xrightarrow{5760} -4{,}1^\circ;\ [M]_D^{20} = -15{,}2^\circ \xrightarrow{1440} +2{,}2^\circ \xrightarrow{5760} -12{,}3^\circ.$$

Eine Lösung von 0,4210 g Sbst. in Wasser zu 20,04 ml Volumen zeigte:

t:	10	20	60	350	700	1460	2160	3600
$2\,\alpha_D^{20}$:	− 0,97°	− 0,92°	− 0,84°	− 0,77°	− 0,62°	− 0,37°	− 0,22°	± 0,00°
		5020	7900	12250	17910	19420	20870	
		+ 0,07°	+ 0,13°	+ 0,26°	+ 0,36°	+ 0,38°	+ 0,38°	

$$[\alpha]_D^{20} = \xrightarrow{10} -23{,}1^\circ \xrightarrow{19420} +9{,}0^\circ;\ [M]_D^{20} = \xrightarrow{10} -68{,}9^\circ \xrightarrow{19420} +27{,}0^\circ.$$

---

[1] Geschätzt.

Als dann 13,5 ml der Lösung mit 1,5 ml 1-n. Salzsäure ([HCl] = 0,1) versetzt wurde, nahm die Drehung wieder zu gemäss:

t:	5	10	20	50	110	220	410
$2\,\alpha_D^{20}$:	+ 0,54°	+ 0,64°	+ 0,80°	+ 1,05°	+ 1,15°	+ 1,20°	+ 1,20°

$$[\alpha]_D^{20} = \longrightarrow + 31{,}7^\circ;\ [M]_D^{20} = \longrightarrow + 94{,}7^\circ.$$

Eine Lösung von 0,4055 g Sbst. in 0,01-n. Salzsäure zu 20,04 ml Volumen zeigte:

t:	10	20	60	120	240	420	840	1780
$2\,\alpha_D^{20}$:	+ 0,13°	+ 0,18°	+ 0,38°	+ 0,53°	+ 0,61°	+ 0,73°	+ 0,78°	+ 0,78°

$$[\alpha]_D^{20} = \xrightarrow{10} + 3{,}2^\circ \xrightarrow{840} + 19{,}3^\circ;\ [M]_D^{20} = \xrightarrow{10} + 9{,}6^\circ \xrightarrow{840} + 57{,}5^\circ.$$

Nach Versetzen von 11,0 g der Lösung mit 1,1 ml 1-n. Salzsäure ([HCl] = 0,1) veränderte sich die Drehung gemäss:

t:	5	10	20	40	100	280	720
$2\,\alpha_D^{20}$:	+ 0,78°	+ 0,83°	+ 0,88°	+ 1,10°	+ 1,13°	+ 1,17°	+ 1,17°,

$$[\alpha]_D^{20} = \longrightarrow + 31{,}8^\circ;\ [M]_D^{20} = \longrightarrow + 94{,}8^\circ.$$

Andererseits zeigte eine Lösung von 0,2460 g *d*-Glucose in 0,1-n. Salzsäure auf 20,04 ml Volumen gelöst:

t:	5	10	20	50	110	230
$2\,\alpha_D^{20}$:	+ 2,18°	+ 1,96°	+ 1,67°	+ 1,36°	+ 1,28°	+ 1,28°

$$[\alpha]_D^{20}\!: = \longrightarrow + 52{,}1^\circ;\ [M]_D^{20} = \longrightarrow + 93{,}9^\circ.$$

Wie beim *l*-Arabinosebenzhydrazid ist also auch hier eine vollständige hydrolytische Spaltung des Zuckerbenzhydrazids in seine Komponenten eingetreten. In stärker sauren Lösungen verläuft diese Spaltung noch schneller, wie die folgenden Messungen zeigen:

0,4073 g Sbst. in 0,1-n. Salzäure wie oben:

t:	10	15	20	30	60	125	270	750
$2\,\alpha_D^{20}$:	+ 0,40°	+ 0,50°	+ 0,68°	+ 0,81°	+ 1,06°	+ 1,23°	+ 1,26°	+ 1,26°

$$[\alpha]_D^{20} = \longrightarrow + 31{,}0^\circ.$$

0,4042 g Sbst. in 0,5-n. Salzsäure auf 10,00 ml Volumen gelöst zeigten:

t:	10	15	20	30	50	100	400
α_D^{20}:	+ 0,54°	+ 0,76°	+ 0,86°	+ 1,00°	+ 1,23°	+ 1,26°	+ 1,26°

$$[\alpha]_D^{20} = \longrightarrow + 31{,}2^\circ.$$

0,4108 g Sbst. in 1,00-n. Salzsäure auf 10,00 ml Volumen gelöst zeigten:

t:	10	15	25	50	120	250
α_D^{20}:	+ 0,74°	+ 0,95°	+ 1,15°	+ 1,30°	+ 1,32°	+ 1,32°.

$$[\alpha]_D^{20} = \longrightarrow + 32{,}1^\circ.$$

Gegen Eisenchlorid und Formaldehyd verhielt sich das Glucosebenzhydrazid wie das Arabinosebenzhydrazid; als 2 g der Glucoseverbindung in 15 ml heissem Wasser mit 1,0 g Thiobenzhydrazid in 15 ml Alkohol versetzt wurde, entstand ein klares Gemisch, welches am folgenden Tage mit 5 ml 2-n. Salzsäure versetzt wurde. Nach ca. 5 Minuten begann das Gemisch sich zu trüben und bis zum folgenden Tage wurden 1,8 g *d*-Glucosethiobenzhydrazid als farblose, schmale und flache Prismen vom Schmp. 181°–183°, mit 10,18, ber. 10,20, % S und in Pyridin von $[\alpha]_D^{20} = -41{,}1°$ ausgeschieden.

8. *d*-Mannosebenzhydrazid

Als eine Lösung von 3,6 g *d*-Mannose in 10 ml Wasser mit 2,8 g Benzhydrazid in 10 ml heissem Alkohol versetzt wurde, entstand ein klares Gemisch, welches nach einer Woche unverändert war und dann bei der freiwilligen Eindunstung in eine strahlig kristallinische Masse überging. Nach dem Trocknen auf Tonscherben wog diese Masse 5,3 g und schmolz unter Zersetzung bei 169°–171° bzw. 178°–179° (Präp. 1). Nach zweimaliger Behandlung von 4,9 g dieses Produktes während einiger Minuten mit je 60 ml warmem, gew. Alkohol waren 1,1 g davon vom Schmp. 177°–179° bzw. 183°–184° ungelöst verblieben (Präp. 2), während aus den Filtraten 0,4 + 0,2 g flache Prismen vom Schmp. 177°–179° bzw. 183°–184° erhalten wurden (Präp. 3). Bei den Analysen ergaben diese Präparate:

Für $C_{13}H_{18}O_6N_2$ (298,29):

ber.:	C 52,34,	H 6,08,	N 9,39 %,
gef., Präp. 1:	» 52,71,	» 6,11,	» 11,01 »,
» » 2:	» 52,32,	» 6,02,	» 9,42 »,
» » 3:	» —	» —	» 9,37 ».

Vom Präp. 3 wurden 0,4154 g mit 2,09 ml 1-n. Natronlauge und Wasser auf 10,00 ml Volumen gelöst, wonach die Lösung *t* Minuten nach dem Zusatz der Lauge die folgenden Drehungen zeigte:

t:	0	10	15	20	60	120	240	720	1440
α_D^{20}:	− 1,14°[1]	− 1,12°	− 1,11°	− 1,10°	− 1,02°	− 0,87°	− 0,72°	− 0,62°	− 0,62°

$$[\alpha]_D^{20} = -27{,}4° \xrightarrow{720} -14{,}9°;\ [M]_D^{20} = -81{,}9° \xrightarrow{720} -44{,}5°.$$

Das Präp. 2 zeigte in der gleichen Weise $[\alpha]_D^{20} = -26{,}9° \longrightarrow -14{,}1°$.

Nach Erwärmen von 3,6 g *d*-Mannose und 2,8 g Benzhydrazid mit 25 ml Wasser 4 Stunden auf dem Wasserbade veränderte sich die entstandene Lösung beim Erkalten nicht; wie oben lieferte sie zum Schluss 5,4 g eines Produktes vom Schmp. 169°–171° bzw. 176°–178°, mit 9,30, ber. 9,39, % N, und in Natronlauge wie oben zeigte es $[\alpha]_D^{20} = -23{,}0° \longrightarrow -13{,}7°$. Beim Erwärmen wie oben von 4,1 g dieses Produktes mit 60 ml gew. Alkohol wurde ein ungelöster Rest von 2,2 g Gewicht, Schmp. 175°–176° bzw. 180°–182°, mit 9,38 % N und vom Drehungsvermögen $[\alpha]_D^{20} = -26{,}1° \longrightarrow -14{,}6°$ erhalten, während aus dem Filtrate 0,3 g flache Prismen vom Schmp. 176°–178° bzw. 183°–184° und $[\alpha]_D^{20} = -25{,}7° \longrightarrow -14{,}7°$ erhalten wurden.

---

[1] Geschätzt.

9. *d*-Galaktosebenzhydrazid

Beim Versetzen einer Lösung von 3,6 g *d*-Galaktose in 10 ml Wasser mit 2,8 g Benzhydrazid in 10 ml heissem Alkohol entstand ein homogenes Gemisch, welches im Laufe einer Woche 2,8 g mit 5 ml 50%igem Alkohol gewaschene, farblose, kleine Prismen vom Schmp. 173°–175° bzw. 184°–186° (Zersetzung) ausschied.

Für $C_{13}H_{18}O_6N_2$ (298,29):

ber.:	C 52,34,	H 6,08,	N 9,39 %,
gef.:	» 52,16,	» 6,00,	» 9,41 » .

In Natronlauge wie unten zeigte das Produkt $[\alpha]_D^{20} = +1{,}9^\circ \xrightarrow{360} +13{,}2^\circ \xrightarrow{2880} +10{,}8^\circ$. Die Mutterlauge ging bei der freiwilligen Eindunstung zu 3,7 g Gewicht in eine etwas sirupöse Kristallmasse über, welche bei der Behandlung mit 25 ml heissem Alkohol einen ungelösten Rest von 0,9 g Gewicht lieferte. Dieses Produkt schmolz bei 174°–176° bzw. 183°–184° und zeigte in Natronlauge $[\alpha]_D^{20} = +1{,}9^\circ \xrightarrow{360} +13{,}1^\circ \xrightarrow{2880} +10{,}7^\circ$. Aus dem Filtrate wurden beim Erkalten 0,4 g nadelförmige Prismen vom Schmp. 174°–176° bzw. 183°–184° abgesetzt.

Nach 4stündiger Erwärmung auf dem Wasserbade von 3,6 g *d*-Galaktose mit 2,8 g Benzhydrazid und 25 ml Wasser lieferte die entstandene Lösung wie oben zuerst 3,1 g flache Prismen vom Schmp. 174°–176° bzw. 183°–185° und wie gewöhnlich von $[\alpha]_D^{20} = +1{,}9^\circ \xrightarrow{360} +12{,}8^\circ \xrightarrow{2880} +10{,}4^\circ$ und dann 0,6 g vom Alkohol ungelöstes Pulver vom Schmp. 174°–176° bzw. 181°–183° und wie oben von $[\alpha]_D^{20} = +2{,}3^\circ \xrightarrow{360} +13{,}5^\circ \xrightarrow{2880} +11{,}4^\circ$. — Beim Erwärmen während 5 Minuten von 2,5 g des zuerst abgeschiedenen Produktes mit 100 ml gew. Alkohol wurden 1,4 g davon gelöst; aus der Lösung kristallisierten bis zum folgenden Tage 0,5 g flache Prismen vom Schmp. 174°–176° bzw. 184°–186° und mit 9,36, ber. 9,39, % N. Eine Lösung von 0,4194 g dieses Produktes in 2,11 ml 1-n. Natronlauge und Wasser auf 10,00 ml Volumen zeigte *t* Minuten nach dem Zusatz der Lauge:

t:	10	15	20	60	120	240	420	720
α_D^{20}:	+ 0,09°	+ 0,09°	+ 0,11°	+ 0,21°	+ 0,34°	+ 0,50°	+ 0,55°	+ 0,53°
					1440	2880	5760	
					+ 0,48°	+ 0,44°	+ 0,44°	

$$[\alpha]_D^{20} = +2{,}1^\circ \xrightarrow{420} +13{,}1^\circ \xrightarrow{2880} +10{,}5^\circ; \quad [M]_D^{20} = +6{,}4^\circ \xrightarrow{420} +39{,}1^\circ \xrightarrow{2880} +31{,}3^\circ.$$

10. Lactosebenzhydrazid

Beim Erhitzen auf dem Wasserbade von 3,6 g Lactose und 1,4 g Benzhydrazid mit 10 ml Wasser entstand ein homogenes Gemisch, welches sich nach 4 Stunden beim Erkalten und bis zum folgenden Tage nicht veränderte, aber bei der freiwilligen Eindunstung auf 7,5 g Gewicht in eine feuchte Kristallmasse überging. Bei der Behandlung dieser Masse mit 10 ml Alkohol, Absaugen, Waschen mit derselben Menge Alkohol und Trocknen an der Luft lieferte sie 4,2 g Kristallpulver, welches nach Sinterung und unter Zersetzung bei 178°–180° bzw. 182°–184° schmolz und kristallwasserhaltig war:

Für $C_{19}H_{28}O_{11}N_2$, $3\,H_2O = C_{19}H_{34}O_{14}N_2$ (514,48):

ber.:	C 44,35,	H 6,66,	N 5,45,	H_2O 10,51 %,
gef.:	» 44,25,	» 6,64,	» 5,43,	» 10,92 ».[1]

Eine Lösung von 0,6394 g des Produktes (= 0,5722 g wasserfreie Sbst.) in 1,86 ml 1-n. Natronlauge und Wasser auf 10,00 ml Volumen zeigte nach *t* Minuten:

t:	10	15	20	240	420	720	1440
0,947 α_D^{20}:	− 0,06°	− 0,06°	− 0,05°	+ 0,18°	+ 0.28°	+ 0,34°	+ 0,34°

Für wasserfreie Substanz gefunden:

$$[\alpha]_D^{20} = -1{,}0° \xrightarrow{720} +5{,}9°;\ [M]_D^{20} = -5° \xrightarrow{720} +27{,}4°.$$

Eine im Vakuum über Phosphorsäureanhydrid getrocknete Probe schmolz bei 169°–171° bzw. 173°–175° (Zers.) und enthielt:

Für $C_{19}H_{28}O_{11}N_2$ (460,43):

ber.:	C 49,56,	H 6,13,	N 6,08 %,
gef.:	» 49,55,	» 6,11,	» 5,96 ».

In Natronlauge wie oben zeigte die getrocknete Probe nach 600 Minuten $[\alpha]_D^{20} = +6{,}8°$.

Åkarp, Schweden, März 1954.

LITERATURVERZEICHNIS

1. B. Holmberg: Arkiv för kemi *4*, 33 (1952).
2. E. L. Hirst, J. K. N. Jones u. E. A. Woods: Journ. Chem. Soc., London 1947, S. 1048.
3. E. Subaschow: Z. Rüb. *46*, 270 (1896); Chem. Zentralbl. 1896, II, 134.
4. E. Davidis: B. *29*, 2308 (1896).
5. A. W. van der Haar: Anleitung zum Nachweis, zur Trennung und Bestimmung der Monosaccharide und Aldehydsäuren, Berlin 1920.
6. H. Wolff: B. *28*, 160 (1895).
7. C. A. Lobry de Bruyn u. W. Alberda v. Ekenstein: Rec. *14*, 203 (1895).
8. B. Holmberg: Arkiv f. kemi, mineral. o geol. *17 A*, N:o 23 (1944).
9. G. Struve: J. pr. Chem. [2] *50*, 300 (1894).

---

[1] Gewichtsverlust im Vakuum über Phosphorsäureanhydrid.

Tryckt den 29 november 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 54

Vorgelegt am 2. Juni 1954

Hexuronsäuren und Thiobenzhydrazid

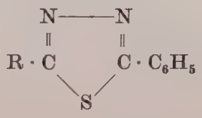
Von Bror Holmberg

Die Fähigkeit des Thiobenzhydrazids, sich mit einfachen Zuckerarten zu schwerlöslichen, durch ein vielseitiges Reaktionsvermögen ausgezeichneten Hydraziden bzw. Hydrazonen umzusetzen[1] hat den Verf. veranlasst, an Hand der *d*-Glucuron-, *d*-Mannuron- und *d*-Galakturonsäuren zu untersuchen, ob das Thiobenzhydrazid auch mit Uronsäuren wohlcharakterisierte Kondensationsprodukte geben kann. Hierbei lieferte die Glucuronsäure ein kristallisiertes Hydrazon[2] des Glucuronsäurelactons (Ia), die Mannuronsäure keine definierbaren Produkte und die Galakturonsäure ein Hydrazon der freien Säure (Ib). Wie bei den entsprechenden Aldose-Verbindungen ergaben die Uronsäure-thiobenzhydrazone bei der Oxydation mit Eisenchlorid die Phenylthiodiazole II a und b.

$R \cdot CH : NNH \cdot CS \cdot C_6H_5$

Ia : $R = C_5H_7O_5$

Ib : $R = C_5H_9O_6$



IIa : $R = C_5H_7O_5$

IIb : $R = C_5H_9O_6$

Zwecks Darstellung des Glucuronsäure-thiobenzhydrazons wurden 3 g *d-Glucuronsäure* in 20 ml Wasser mit einer Lösung von 2,5 g Thiobenzhydrazid in 10 ml 2-n. Salzsäure versetzt. Hierbei entstand ein homogenes Gemisch, welches bald emulgiert wurde, und nach dem Impfen mit einer bei einem Vorversuche als kristallinische Bodenkruste erhaltenen Probe wurden nach 6 Stunden beim Absaugen, Waschen mit 20 ml Wasser, 10 ml Alkohol und 10 ml Äther 4,7 g nadelförmige Prismen vom Schmp. 167°–169° (unter Gasentwicklung in einer gelbbraunen Schmelze) gewonnen, welche gemäss der Analyse aus dem Thiobenzhydrazon des Glucurons bestanden.

Für $C_{13}H_{14}O_5N_2S$ (310,32):

ber.:	C 50,31,	H 4,55,	N 9,03,	S 10,33 %,
gef.:	» 50,35,	» 4,54,	» 9,00,	» 10,32 ».

Eine in der Wärme bereitete Lösung von 0,3713 g Sbst. in 20 ml 50%igem Alkohol verblieb beim Erkalten unverändert und ergab dann mit Phenolphthalein und 4,80 ml 0,1000-n. Natronlauge einen unscharfen Umschlag. Die Farbe verschwand indessen schnell und nach 60 Minuten wurden noch 1,25, dann nach 30 Minuten 1,00 ml der Lauge verbraucht und erst nach dem Zusatz von insgesamt 20,25 ml Lauge war die letzte, durch 0,10 ml Lauge hervorgerufene Färbung noch am folgenden Tage sichtbar. Entfärbung trat dann mit 0,25 ml 0,1000-n. Salzsäure ein, aber die rote Farbe kam bald wieder zurück und nach 10 Minuten wurden 0,20 ml der Säure verbraucht. Nach dem Zusatz von noch 10,00 ml der Säure wurde am folgenden Tage die Lösung vorübergehend durch 4,80 ml der Lauge gerötet.

---

[1] Arkiv f. kemi *4*, 33 (1952).

[2] Um eine Verwechselung mit den entsprechenden Säurehydraziden zu vermeiden, werden die hier erhaltenen Verbindungen als Hydrazone benannt und formuliert.

Eine Lösung von 0,5032 g Sbst. in Pyridin auf 10,00 ml Volumen zeigte 10 bzw. 60 und 420 Min. nach dem Zusatz des Lösungsmittels

$$\alpha_D^{20} = -1°,19,\ [\alpha]_D^{20} = -23°,6 \text{ und } [M]_D^{20} = -73,4°.$$

Beim Schütteln von 1,3 g *d*-Glucuron-thiobenzhydrazon mit 10 ml 1-n. Natronlauge und 10 ml Alkohol entstand schnell eine gelbrote Lösung, welche am folgenden Tage braunrot war und dann durch 5 ml 2-n. Salzsäure nur schwach getrübt wurde und bei freiwilliger Eindunstung ein rotbraunes, klebriges Öl ausgeschieden hatte.

Als eine wie oben aus 3,3 g Glucuron-thiobenzhydrazon, 25 ml 0,5-n. Natronlauge und 25 ml Alkohol hergestellte Lösung mit 50 ml Wasser verdünnt und dann mit 11 ml 2-m. Eisenchlorid-Lösung versetzt wurde trat sogleich eine schwache Wärmeentwicklung ein und eine geringe Menge eines teigigen Pulvers wurde abgeschieden. Das Filtrat davon wurde durch 10 ml 2-n. Salzsäure nicht verändert und lieferte dann bei der freiwilligen Eindunstung auf 33 g Gewicht 2 g Kristallwarzen vom Schmp. ca. 177°–180° (unter Zersetzung). Von diesem Produkte wurden 1,8 g aus 100 ml gew. Alkohol umkristallisiert, wobei 0,9 g reines Oxydationsprodukt als schmale Prismen vom Schmp. 198°–199° (Zersetzung) erhalten wurden.

Für $C_{13}H_{12}O_5N_2S$ (308,31):

ber.:	C 50,64,	H 3,92,	N 9,09,	S 10,40 %,
gef.:	» 50,50,	» 3,89,	» 9,15,	» 10,41 ».

Eine Lösung von 0,2135 g Sbst. in Pyridin auf 10,00 ml Volumen zeigte nach 10 bzw. 50 und 150 Minuten nach dem Zusatz des Pyridins $\alpha_D^{20} = -1,37°$, $[\alpha]_D^{20} = -64,2°$ und $[M]_D^{20} = -198°$.

Als 5 g eines von *Delta Chemical Works, New York*, bezogenen, glasigen, als »*Mannuronic Acid (Ba Salt)*« angegebenen Präparates mit 20 ml 1-n. Salzsäure behandelt wurden, entstand unter Gasentwicklung eine braune Lösung, welche von 3 g Thiobenzhydrazid in 11 ml 2-n. Salzsäure emulgiert wurde und dann bis zum folgenden Tage ein klebriges, dunkelbraunes Öl ausschied. Aus diesem Produkte konnte nichts kristallisierbares isoliert werden, und als die Mutterlauge mit 5 g Natriumazetat in 10 ml Wasser versetzt wurde, entstand eine Emulsion, welche 0,7 g Thiobenzhydrazid vom Schmp. 79°–81° absetzte.

Ein Gemisch aus 4,0 g *d-Galakturonsäure* in 15 ml Wasser und 3,0 g Thiobenzhydrazid in 25 ml 1-n. Salzsäure begann nach einigen Minuten ein Kristallpulver auszuscheiden, und am folgenden Tage wurden beim Absaugen und Waschen mit 10 ml Wasser 6,1 g *d*-Galakturonsäure-thiobenzhydrazon vom Schmp. 144°–145° (unter Gasentwicklung in einer braunen Schmelze) erhalten.

Für $C_{13}H_{16}O_6N_2S$ (328,34):

ber.:	C 47,55,	H 4,91,	N 8,53,	S 9,77 %,
gef.:	» 47,33,	» 4,95,	» 8,53,	» 9,69 ».

0,3221 g Sbst. ergaben mit Wasser, Phenolphthalein und 10,65 ml 0,1000-n. Natronlauge eine klare, schwach rosafarbene Lösung, Äquiv.-Gew. ber. 328,3, gef. 303.

In Pyridin auf 10,00 ml Volumen gelöst zeigten 0,5148 g Sbst. nach t Minuten:

t:	15	30	120	240	420
α_D^{20}:	$-3,56°$	$-3,54°$	$-3,49°$	$-3,47°$	$-3,47°$

$[\alpha]_D^{20} = \xrightarrow{15} -69,2° \xrightarrow{240} -67°,4$ und $[M]_D^{20} = \xrightarrow{15} -227° \xrightarrow{240} -221°$.

Beim Umkristallisieren von 1,0 g obigen Produktes aus 25 ml Wasser wurden 0,7 g eines Kristallpulvers erhalten, welches bei 142°–143° (Zersetzung) schmolz, 9,81, ber. 9,77, % S enthielt und in Pyridin wie oben $[\alpha]_D^{20} = \xrightarrow{15} -69,6° \xrightarrow{240} -62,8°$ zeigte. — Als andererseits 1,0 g des Produktes in 25 ml heisser, 1-n. Salzsäure 10 Minuten auf dem Wasserbade erhitzt wurde, wurde die Lösung bald durch ein braunes Pulver getrübt und das Filtrat davon schied beim Erkalten eine graubraune, klebrige Masse aus.

Als 3,2 g des rohen Galakturonsäure-thiobenzhydrazons mit 22 ml 0,5-n. Natronlauge geschüttelt wurden, entstand ein fast gelatinöser Brei, welcher beim Zusatz von 20 ml Wasser eine klare Lösung lieferte. Diese wurde mit 10 ml 2-m. Eisenchlorid-Lösung versetzt, wobei ein Brei aus einem feinkristallinischen Pulver entstand, welcher mit 5 ml 2-n. Salzsäure versetzt wurde, wonach er am folgenden Tage 2,9 g auf Tonscherben getrocknetes, weisses Pulver ergab. Dieses schmolz bei 180°–182° (Zersetzung) und bestand gemäss der Analyse aus etwas wasserhaltigem 5-Carboxytetraoxybutyl-2-phenylthiodiazol-1,3,4.

Für $C_{13}H_{14}O_6N_2S$, $0,89\ H_2O$ (342,36):

ber.:	C 45,60,	H 4,65,	N 8,18,	S 9,37,	H_2O 4,68 %,
gef.:	» 45,71,	» 4,50,	» 8,38,	» 9,58,	» 4,68 ».

Eine Lösung von 0,3397 g Sbst. in warmem Wasser ergab einen guten Umschlag mit Phenolphthalein und 9,85 ml 0,1000-n. Natronlauge, Äquiv.-Gew. ber. 342,4, gef. 344,9.

Eine Lösung von 0,4132 g Sbst. in Pyridin zu 20,04 ml Volumen zeigte sogleich und nach 70 Minuten 2 $\alpha_D^{20} = +0,06°$, $[\alpha]_D^{20} = +1,5°$.

Diese Untersuchung wurde in der damaligen org.-chem. Abteilung des Nobelinstitutes der Kgl. Akademie der Wissenschaften, Stockholm, ausgeführt. Die Analysen verdankt der Verf. Frau MAJBRITT VESTERGAARD.

Åkarp, Schweden, April 1954.

Tryckt den 29 november 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 55

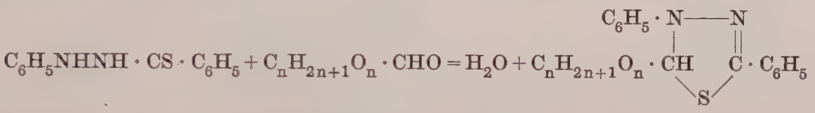
Vorgelegt am 2. Juni 1954

Zur Kenntnis der Glykothiodiazoline

Von Bror Holmberg

1. Einleitung und Übersicht

Bei vorsichtigen Erhitzungen von Aldosen mit α-Phenyl-β-thiobenzoylhydrazin (2-Phenylthiobenzhydrazid) in etwas chlorwasserstoffhaltigem Alkohol erhielt Wuyts (1) gemäss der Bruttogleichung


$C_6H_5NHNH \cdot CS \cdot C_6H_5 + C_nH_{2n+1}O_n \cdot CHO = H_2O + C_nH_{2n+1}O_n \cdot CH$
$C_6H_5 \cdot N$
N
S
$C \cdot C_6H_5$

5-Polyoxyalkyl-2,4-diphenyl-4,5-dihydrothiodiazole-1,3,4, welche er als *Glykothiodiazoline* bezeichnete und von denen er erwartete, dass sie auf Grund ihrer leichten Bildung und hohen Drehungen als Hilfsmittel bei der Analyse und den Studien der Zuckerarten nützlich sein könnten (2). Eine fühlbare Einschränkung in der Verwendung zu diesen Zwecken erleiden indessen die Glykothiodiazoline durch das gleichzeitige Entstehen von wenigstens zwei wahrscheinlich stereoisomeren Formen von stark verschiedenem Drehungsvermögen, deren vollständige Trennung nicht gelang.

Bei fraktionierten Behandlungen der direkt erhaltenen Glykothiodiazoline mit Gemischen aus Methylalkohol (bei dem Arabinoglykothiodiazolin Azeton) und Pyridin erhielt Wuyts (3 und 4) Lösungen, aus deren Konzentrationen und Drehungen die in der Tabelle 1 angegebenen Werte der sp. Drehungsvermögen der am Anfang und zum Schluss herausgelösten Anteile berechnet wurden.

Tabelle 1.

Glykothiodiaz. der	$[\alpha]^{20}\ \lambda 575$	
Arabinose	$+763{,}5°$	$+228°$
Xylose	$-1153°$	$+1651°$
Rhamnose	$+991°$	$-1022°$
Fucose	$-1030°$	$-70{,}5°$
Glucose	$-406{,}6°$	$+290{,}5°$
Mannose	$-233{,}6°$	$+970°$
Galaktose	$-568{,}2°$	$+1406°$

Um zu untersuchen, ob es an Hand der bei den Zucker-Verbindungen des Thiobenzhydrazids selbst gemachten Erfahrungen (5) möglich wäre, zu einem tieferen Einblick in die Chemie der Glykothiodiazoline zu kommen, wurden vom

Verf. eine Anzahl solcher Verbindungen durch Umsetzen bei gew. Temperatur von Aldosen mit 2-Phenylthiobenzhydrazid in mit verd. Salzsäure versetztem Alkohol dargestellt. Die hierbei nach verschiedenen Zeiten isolierten Produkte zeigten die in der Tabelle 2 angegebenen Schmelzpunkte und sp. Drehungsvermögen in Pyridin bei c = ca. 2,5 g/100 ml.

Tabelle 2.

Glykothiodiazolin der	Fraktion	Schmp.	$[\alpha]^{20}_{D}$
l-Arabinose	1	220°—223°	+ 711°
»	2	213°—215°	+ 619°
d-Xylose	1	163°—164°	+ 1435°
»	2	162°—163°	+ 1440°
»	3	160°—161°	+ 1403°
»	4	125°—128°	− 653°
d-Lyxose[1]	1	151°—153°	+ 936°
»	2	151°—153°	+ 977°[2]
d-Ribose[1]	1	170°—171°	− 1007°
»	2	162°—165°	− 964°[3]
l-Rhamnose[1]	1	175°—176°	− 936°
»	2	165°—167°	− 861°
»	3	166°—168°	+ 854°
l-Fucose	1	193°—194°	− 624°
d-Glucose	1	147°—149°	+ 882°[4]
»	2	143°—146°	+ 42°[2]
»	3	135°—140°	− 287°
d-Mannose[1]	1	201°—202°	+ 743°
»	2	200°—202°	+ 69,6°
»	3	201°—202°	+ 840°[5]
»	4	197°—200°	+ 879°
»	5	187°—192°	(− 234°)
d-Galaktose	1	179°—181°	+ 1109°
»	2	176°—180°	− 114°
»	3	176°—180°	+ 598°
»	4	170°—175°	+ 73°

Die Pyridinlösungen der Glykothiodiazoline zeigten wenigstens während der ersten Stunden konstantes Drehungsvermögen, aber über längere Zeiten ausgestreckte Messungen von Präparaten des *d*-Galaktothiodiazolins ergaben deutliche, numerische Abnahmen. So zeigte z. B. ein Präparat von $[\alpha]^{20}_{D} = +1059°$ nach 24 Stunden $[\alpha]^{20}_{D} = +1051°$ und nach 76 Stunden + 1015°, während ein Präparat von $[\alpha]^{20}_{D} = -128°$ nach 25,5 Stunden − 124° und nach 66 Stunden − 115° ergab.

Erhitzungen von Glykothiodiazolinen während *t* Minuten bei *T*° C oder auf dem Wasserbade (W-B) ohne oder mit Zusätzen von 1-n. Salzsäure oder 0,5-n. Natronlauge ergaben die aus der Tabelle 3 ersichtlichen Drehungsänderungen.

---

[1] Enthält 1 Mol. H_2O.

[2] Aus gew. Alkohol umkristallisiert.

[3] Nach Umkristallisierung aus gew. Alkohol Schmp. 169°–171° und $[\alpha]^{20}_{D} = -1060°$.

[4] » » » » » » 154°–155° und $[\alpha]^{20}_{D} = +1233°$.

[5] » « » » » » 198°–200° und $[\alpha]^{20}_{D} = +894°$.

Tabelle 3.

Glykothiodiaz. der	Zusatz	t	$T°$	$[\alpha]^{20}_{D}$
l-Arabinose	—	—	—	+ 711°
»	—	60	150	+ 701°
»	—	60	175	+ 644°
»	—	120	175	+ 627°
»	HCl	240	W-B	+ 450°
»	NaOH	240	W-B	+ 341°
d-Xylose	—	—	—	+ 1440°
»	—	120	140—145	+ 1320°[1]
d-Mannose	—	—	—	+ 69,6°
»	—	10	190	+ 856°
»	—	20	200—203	+ 887°[2]

Aus den Tabellen geht hervor, dass die Drehungsverhältnisse der Glykothiodiazoline sehr verwickelt sind und da es ausserdem dem Verf. nicht gelungen ist, diese Verbindungen in besser charakterisierbare Derivate zu überführen, wurde die Untersuchung nicht weiter fortgesetzt.

Diese Untersuchung wurde in der damaligen org.-chemischen Abteilung des Nobelinstitutes der Kgl. Akademie der Wissenschaften, Stockholm, ausgeführt. Für die Ausführungen der Elementaranalysen hat der Verf. besonders Frau MAJBRITT VESTERGAARD und für sonstige Mithilfe dem Hrn. Lic. Phil. JAN SANDSTRÖM bestens zu danken.

2. 2-Phenylthiobenzhydrazid

Das 2-Phenylthiobenzhydrazid, welches WUYTS und Mitarbeiter durch Umsatz der Dithiobenzoesäure mit Phenylhydrazin darstellten und für welches sie den Schmp. 88° (6) bzw. 88,4° (7) fanden, wurde vom Verf. in der analogen Weise aus der Thiobenzoylthioglykolsäure und Phenylhydrazin gewonnen. Hierbei wurden 21,2 g dieser Säure mit Natriumhydroxyd in 75 ml Wasser neutralisiert und die Lösung mit 12 g Phenylhydrazin in dem gleichen Volumen Alkohol versetzt, wobei ein homogenes Gemisch entstand. Nach etwa 15 Minuten trat Emulgierung ein, und nach dem Impfen mit einer bei einem Vorversuche erhaltenen, kristallisierten Probe wurden bis zum folgenden Tage 21,5 g mit 50 ml 50%igem Alkohol gewaschene, flache, blassgelbe Nadeln vom Schmp. 89°–90° ausgeschieden.

Für $C_{13}H_{12}N_2S$ (228,31):

ber.:	C 68,38,	H 5,30,	N 12,27,	S 14,05 %,
gef.:	» 68,30,	» 5,31,	» 12,25,	» 14,00 ».

Beim Umkristallisieren von 18 g des Rohproduktes aus 50 ml Benzol wurden 12 g flache, schwefelgelbe Prismen oder schmale Täfelchen vom Schmp. 89,5°–90,5° erhalten; aus der Mutterlauge wurden mit der doppelten Menge Petroläther noch 4,2 g blassgelbe Schuppen vom Schmp. 89°–90° ausgefällt.

Als eine Lösung von 2,3 g 2-Phenylthiobenzhydrazid in 20 ml warmem Alkohol mit 2,5 ml 5-n. Salzsäure versetzt wurde, trat auch beim Erkalten keine Veränderung ein und bei der freiwilligen Eindunstung der Lösung wurden 2,2 g des Thiohydrazids vom Schmp. 89°–90° zurückgewonnen. — Eine Lösung von 0,3110 g des 2-Phenylthiobenzhydrazids in 10 ml Alkohol ergab einen guten Umschlag mit Phenolphthalein und 13,57 ml 0,1000-n. Natronlauge; die Lösung, welche bald milchig wurde, wurde mit 40 ml Wasser verdünnt, und nach dem Zusatz von Stärke-Lösung wurde sie von 14,10 ml 0,0972-n. Jod-Lösung braungrün gefärbt, Äquiv.-Gew. ber. 228,3, gef. 229,2 bzw. 226,9.

---

[1] Aus Wasser umkristallisiert.

[2] Aus gew. Alkohol umkristallisiert.

Als eine Lösung von 2,3 g des 2-Phenylthiobenzhydrazids in 10 ml 1-n. Natronlauge an der Luft verwahrt wurde, wurde ein rotgelbes Kristallpulver ausgeschieden, welches ab und zu abgesaugt wurde; im Laufe eines Monates wurden in dieser Weise insgesamt 1,9 g Oxydationsprodukt vom Schmp. (unter Zersetzung) 128°–130° bei langsamer und 137°–138° bei schneller Erhitzung (Schmelze blasig) erhalten. Beim Umkristallisieren aus Benzol lieferte dieses aus dem Disulfid des 2-Phenyl-*iso*-thiobenzhydrazids bestehende Produkt gelbrote, schmale Prismen vom Schmp. wie oben 135°–136° bzw. 141°–142° (unter Zersetzung).[1]

Für $C_{26}H_{22}N_4S_2$ (454,60):

ber.:	C 68,69,	H 4,88,	N 12,33,	S 14,10 %,
gef.:	» 68,80,	» 4,90,	» 12,36,	» 14,07 ».

Als eine Lösung von 3,4 g 2-Phenylthiobenzhydrazid in 30 ml Alkohol mit 1,5 g *Formalin* und 3 ml 5-n. Salzsäure versetzt wurde, trat eine schwache Wärmeentwicklung und Emulgierung ein; bis zum folgenden Tage entstand ein Brei aus 3,3 g eines mit 10 ml Alkohol gewaschenen, blassgelben, grobkristallinischen Pulvers vom Schmp. 94°–96°[2] und der Zusammensetzung des erwarteten 2,4-Diphenyl-4,5-dihydrothiodiazols-1,3,4.

Für $C_{14}H_{12}N_2S$ (240,32):

ber.:	C 69,97,	H 5,03,	N 11,66,	S 13,34 %,
gef.:	» 70,00,	» 5,08,	» 11,65,	» 13,26 ».

Das entsprechende 2,4,5-Triphenylthiodiazolin wurde erhalten, als eine Lösung von 2,3 g 2-Phenylthiobenzhydrazid in 20 ml Alkohol mit 1,1 g *Benzaldehyd* und 2 ml 5-n. Salzsäure versetzt wurde. Hierbei trat schwache Wärmeentwicklung ein und ein Öl wurde abgeschieden, welches in 2,9 g einer aus schmalen, blassgelben Prismen bestehenden Kristallmasse vom Schmp. 96°–98°[2] überging.

Für $C_{20}H_{16}N_2S$ (316,41):

ber.:	C 75,91,	H 5,10,	N 8,86,	S 10,13 %,
gef.:	» 75,69,	» 5,03,	» 8,93,	» 10,10 ».

3. *l*-Arabinothiodiazolin

Eine Lösung von 1,5 g *l*-Arabinose in 5 ml 2-n. Salzsäure wurde am folgenden Tage mit 2,3 g 2-Phenylthiobenzhydrazid in 20 ml Alkohol versetzt, wonach das klare Gemisch nach *t* Minuten die folgenden Drehungen zeigte:

---

[1] Für ein aus dem 2-Phenylthiobenzhydrazid durch Luftoxydation entstandenes Disulfid geben Wuyts und Lacourt (8) den Schmp. 145° an, während sie für ein durch Oxydation mit Jod gewonnenes den Schmp. 149° fanden.

[2] Wuyts und Lacourt (9) fanden sowohl für das Diphenyl- wie für das Triphenylthiodiazolin den Schmp. 97°.

t:	3	5	8	13	20
α_D^{20}:	+4,48°	+5,04	+5,77°	+7,02°	+8,47°.

Die Lösung begann dann Kristalle abzuscheiden und am folgenden Tage wurden 3,0 g mit 10 ml Alkohol gewaschene, cremefarbene, fast haarfeine Nadeln vom Schmp. 220°–222°[1] erhalten.

Für $C_{18}H_{20}O_4N_2S$ (360,42):

ber.:	C 59,98,	H 5,59,	N 7,77,	S 8,90 %,
gef.:	» 59,96,	» 5,66,	» 7,83,	» 8,95 ».

Eine Lösung von 0,2529 g der analysierten Sbst. in Pyridin auf 10,04 ml Volumen gelöst zeigte sofort und nach 2 Stunden $\alpha_D^{20} = +17{,}60°$, $[\alpha]_D^{20} = +699°$.

Die Mutterlauge des obigen Produktes ergab bei der freiwilligen Eindunstung noch 0,4 g Nadeln vom Schmp. 216°–218° und wie oben von $[\alpha]_D^{20} = +654°$.

Bei einem neuen Versuche wurde eine Lösung von 3,0 g *l*-Arabinose in 10 ml 1-n. Salzsäure sofort mit 4,6 g 2-Phenylthiobenzhydrazid in 50 ml Alkohol versetzt, wonach bis zum folgenden Tage 5,7 g haarfeine Nädelchen vom Schmp. 220°–223°, mit 59,92 % C, 5,56 % H, 7,84 % N und 8,82 % S ausgeschieden wurden. Dieses Produkt zeigte in Pyridin $[\alpha]_D^{20} = +711°$, und bei der freiwilligen Eindunstung der Mutterlauge auf 20 g Gewicht wurden noch 1,1 g Nädelchen vom Schmp. 213°–215° und $[\alpha]_D^{20} = +619°$ erhalten. Bei der Behandlung von 0,8 g von diesem Produkte mit 50 ml heissem, gew. Alkohol wurden 0,3 g davon gelöst. Der ungelöste Teil schmolz bei 216°–218° und zeigte $[\alpha]_D^{20} = +621°$, während beim Erkalten der Lösung 0,1 g Nadeln vom Schmp. 221°–222° und $[\alpha]_D^{20} = +698°$ auskristallisierten.

Nach einstündiger Erhitzung bei 150° einer Probe des Produktes mit $[\alpha]_D^{20} = +711°$ zeigte diese den Schmp. 221°–223° und $[\alpha]_D^{20} = +701°$, während nach den gleichen Behandlungen bei 175° nach 1 bzw. 2 Stunden die Schmp. 219–221° bzw. 219°–221° und $[\alpha]_D^{20} = +644°$ bzw. $+627°$ gefunden wurden. — Nach 4-stündiger Erhitzung auf dem Wasserbade und unter gelegentlichen Umschüttelungen von 1,0 g desselben Produktes mit 50 ml 1-n. Salzsäure wurde die gleiche Menge Substanz zurückgewonnen; diese schmolz bei 220°–222°, enthielt 60,02 % C, 5,59 % H, 7,85 % N, 8,95 % S und zeigte $[\alpha]_D^{20} = +450°$. — Bei der gleichen Behandlung von 1,0 g Sbst. mit 20 ml 0,5-n. Natronlauge wurden 0,8 g eines ungelösten Pulvers vom Schmp. 219°–221° und $[\alpha]_D^{20} = +341°$ erhalten.

Oxydationen des *l*-Arabinothiodiazolins mit Eisenchlorid, Kaliumpersulfat und Pyridiniumtribromid ergaben keine definierbaren Produkte.

4. *d*-Xylothiodiazolin

Eine Lösung von 3,0 g *d*-Xylose in 6 ml Wasser wurde am folgenden Tage mit 4,0 g 5-n. Salzsäure und 4,6 g 2-Phenylthiobenzhydrazid in 60 ml Alkohol versetzt, wonach das Gemisch nach t Minuten die folgenden Drehungen zeigte:

t:	2	5	10	20	40	80	150	270	450
α_D^{20}:	+1,54°	+1,72°	+2,12°	+2,82°	+4,10°	+6,28°	+9,34°	+13,24°	+17,20°.

Dann begann Kristallisation und am folgenden Tage wurden beim Absaugen und Waschen mit 5 ml Alkohol 2,0 g schwach gelbliche, schmale und flache Prismen vom Schmp. 163°–164° (Präp. 1) erhalten. Das Filtrat schied dann im Laufe von 5 Tagen noch 1,4 g Kristalle vom Schmp. 162°–163° (Präp. 2) aus und das neue Filtrat lieferte bei der freiwilligen Eindunstung Effloreszenzen, welche sich in Wärme lösten, wonach beim Erkalten 1,6 g cremefarbene, schmale Prismen vom Schmp. 160°–161° (Präp. 3) ausgeschieden wurden. Das zuletzt erhaltene Filtrat wurde mit dem gleichen Volumen Wasser versetzt, wobei es emulgiert wurde und dann 1,9 g nadelförmige Prismen vom Schmp. 125°–128° (Präp. 4) ausschied.

---

[1] Gemäss Wuyts (1) 222°.

Die Analysen der verschiedenen Präparate ergaben:

Für $C_{18}H_{20}O_4N_2S$ (360,42):

ber.:	C 59,98,	H 5,59,	N 7,77,	S 8,90 %,
gef., Präp. 1:	» 60,46,	» 5,60,	» 7,78,	» 8,93 »
» » 2:	» 59,87,	» 5,56,	» 7,84,	» 8,97 »
» » 3:	» 59,91,	» 5,64,	» 7,74,	» 8,87 »
» » 4:	» 60,09,	» 5,59,	» 7,93,	» 9,19 ».

Vom Präp. 1 zeigten 0,3430 g in Pyridin auf 10,04 ml Volumen gelöst sofort und nach 4 Stunden 0,5 $\alpha_D^{20} = +24{,}54°$, $[\alpha]_D^{20} = +1435°$; von den anderen Produkten zeigten in der gleichen Weise Präp. 2: $[\alpha]_D^{20} = +1440°$, Präp. 3: $[\alpha]_D^{20} = +1403°$ und Präp. 4: $[\alpha]_D^{20} = -653°$.

Beim Umkristallisieren des Präp. 1 ergaben 1,5 g mit 25 ml heissem Wasser eine homogene Lösung, welche beim Erkalten 1,0 g blassgelbe Prismen vom Schmp. 161°–163° und mit $[\alpha]_D^{20} = +1454°$ lieferte. — Vom Präp. 2 lieferten 1,0 g beim Umkristallisieren aus 15 ml gew. Alkohol 0,7 g schwach cremefarbene, schmale und dünne Täfelchen vom Schmp. 162°–164°, mit 8,88, ber. 8,90, % S und in Pyridin von $[\alpha]_D^{20} = +1450°$. — Als 0,3 g vom Präp. 2 während 2 Stunden bei 140°–145° erhitzt wurden, entstand eine rotbraune Schmelze, welche beim Erkalten glasig erstarrte und beim Kristallisieren aus 2 ml Wasser 0,1 g haarfeine Nädelchen vom Schmp. 148°–150° und $[\alpha]_D^{20} = +1320°$ lieferte.

Gegen Oxydationsmittel verhielt sich das Xylothiodiazolin wie das entsprechende Arabinose-Derivat.

5. *d*-Lyxothiodiazolin

Eine Lösung von 1,6 g *d*-Lyxose in 5 ml 2-n. Salzsäure wurde am folgenden Tage mit 2,5 g 2-Phenylthiobenzhydrazid in 22 ml Alkohol versetzt, wonach das homogene Gemisch nach *t* Minuten die folgenden Drehungen zeigte:

t:	5	10	20	40	80	160	300	600
α_D^{20}:	−0,87°	−1,27°	−1,82°	−2,60°	−3,77°	−5,12°	−6,02°	−5,52°.

Danach begann Kristallisation, worauf bis zum folgenden Tage 2,0 g mit 10 ml 50 % igem Alkohol gewaschene, cremefarbene, nadelförmige Prismen vom Schmp. 151°–153° ausgeschieden wurden.

Für $C_{18}H_{20}O_4N_2S, H_2O = C_{18}H_{22}O_5N_2S$ (378,44):

ber.:	C 57,13,	H 5,86,	N 7,40,	S 8,47 %,
gef.:	» 57,18,	» 5,93,	» 7,43,	» 8,42 ».

Eine Lösung von 0,2022 g der Sbst. in Pyridin auf 10,00 ml Volumen zeigte sofort und nach 5 Stunden $\alpha_D^{20} = +18{,}93°$, $[\alpha]_D^{20} = +936°$.

Aus der Mutterlauge des obigen Produktes wurden bei freiwilliger Eindunstung auf 13 g Gewicht noch 1,3 g Kristalle vom Schmp. ca. 110°–115° gewonnen, welche bei der Umkristallisierung aus 10 ml gew. Alkohol 0,5 g cremefarbene, nadelförmige Prismen vom Schmp. 151°–153° lieferten. Dieses Präparat enthielt 8,57, ber. 8,47, % S und zeigte in Pyridin wie oben $[\alpha]^{20}_{D} = +977°$.

6. *d*-Ribothiodiazolin

Eine Lösung von 3,0 g *d*-Ribose in 10 ml 2-n. Salzsäure wurde am folgenden Tage mit 4,6 g 2-Phenylthiobenzhydrazid in 40 ml Alkohol versetzt, wonach das klare Gemisch die folgenden Drehungen zeigte:

t:	10	15	20	40	80	160	360	720
$0,5\ \alpha^{20}_{D}$:	−0,82°	−0,87°	−0,92°	−1,37°	−2,12°	−3,97°	−8,52°	−15,92°.

Danach begann Kristallisation, worauf nach 2 Tagen 5,0 g mit 10 ml 50 % igem Alkohol gewaschene, schmale, gelbliche Nadeln vom Schmp. 170°–171° ausgeschieden wurden.

Für $C_{18}H_{20}O_4N_2S,\ H_2O = C_{18}H_{22}O_5N_2S$ (378,44):

ber.:	C 57,13,	H 5,86,	N 7,40,	S 8,47 %,
gef.:	» 56,98,	» 5,86,	» 7,39,	» 8,52 ».

Eine Lösung von 0,2066 g der analysierten Substanz in Pyridin auf 10,00 ml Volumen zeigte sofort und nach 3 Stunden $\alpha^{20}_{D} = -20,80°$, $[\alpha]^{20}_{D} = -1007°$.

Aus der Mutterlauge des obigen Produktes wurden bei freiwilliger Eindunstung auf 13 g Gewicht noch 1,5 g einer Kristallmasse erhalten, welche bei 162°—165° schmolz und in Pyridin $[\alpha]^{20}_{D} = -964°$ zeigte. Beim Umkristallisieren von 1,2 g davon aus 10 ml gew. Alkohol wurden 0,7 g schmale Prismen vom Schmp. 169°—171° und $[\alpha]^{20}_{D} = -1060°$ (in Pyridin) erhalten.

7. *l*-Rhamnothiodiazolin

Eine Lösung von 1,85 g *l*-Rhamnose in 5 ml 0,8-n. Salzsäure wurde am folgenden Tage mit 2,3 g 2-Phenylthiobenzhydrazid in 30 ml Alkohol versetzt, wonach das Gemisch nach *t* Minuten die folgenden Drehungen zeigte:

t:	10	20	40	90	180	360	720	1410	2850	4950
α^{20}_{D}:	+0,18°	+0,23°	+0,37°	+0,66°	+1,05°	+1,65°	+2,09°	+1,42°	−2,82°	−10,58°.

Darauf zeigten sich bald kleine, haarfeine Nadeln und am folgenden Tage wurden beim Absaugen und Waschen mit 5 ml Alkohol 1,1 g solche Kristalle vom Schmp. 175°–176° gewonnen.

Für $C_{19}H_{22}O_4N_2S,\ H_2O = C_{19}H_{24}O_5N_2S$ (392,46):

ber.:	C 58,14,	H 6,16,	N 7,14,	S 8,17 %,
gef.:	» 58,59,	» 6,16,	» 7,16,	» 8,15 ».

Eine Lösung von 0,2248 g des analysierten Produktes in Pyridin auf 10,04 ml Volumen zeigte sofort und nach 2 Stunden $0,5\ \alpha^{20}_{D} = -10,48°$, $[\alpha]^{20}_{D} = -936°$.

Das Filtrat des obigen Produktes schied im Laufe einer Woche noch 0,55 g Kristalle vom Schmp. 165°–167°, mit 7,19, ber. 7,14, % N und 8,21, ber. 8,17, % S, und in Pyridin von $[\alpha]^{20}_{D} = -861°$, aus. Beim Versetzen des Filtrates dieses Produktes mit 40 ml Wasser entstand eine Emulsion, welche 1,65 g cremefarbene Nadeln vom Schmp. 166°–168° ausschied. Dieses Produkt schmolz bei 166°–168°, enthielt wie die vorigen 1 H_2O:

ber.:	C 58,14,	H 6,16,	N 7,14,	S 8,17 %,
gef.:	» 58,07,	» 6,12,	» 7,29,	» 8,45 »,

und zeigte in Pyridin wie gewöhnlich $[\alpha]_D^{20} = +854°$.

8. *l*-Fucothiodiazolin

Eine Lösung von 0,65 g *l*-Fucose in 2,5 ml 2-n. Salzsäure wurde am folgenden Tage mit 1,2 g 2-Phenylthiobenzhydrazid in 10 ml Alkohol versetzt, wonach das Gemisch die folgenden Drehungen zeigte:

t:	5	10	20	40	80
α_D^{20}:	−4,52°	−5,26°	−6,36°	−8,77°	−12,27°,

wonach Kristallisation begann; bis zum folgenden Tage entstand ein Brei aus 1,4 g blassgelber Nadeln vom Schmp. ca. 182°–185°. Das Produkt enthielt 8,15 % N und 9,07 % S und zeigte in Pyridin wie unten $[\alpha]_D^{20} = -624°$. Beim Schütteln von 0,7 g davon eine Stunde mit 10 ml 0,5-n. Natronlauge lösten sich 0,15 g, während der mit Wasser und Äther gewaschene, ungelöste Rest bei 193°–194° schmolz.

Für $C_{19}H_{22}O_4N_2S$ (374,45):

ber.: N 7,48, S 8,56 %; gef.: N 7,67, S 8,57 %.

Von dem gereinigten Produkte zeigten 0,2007 g in Pyridin auf 10,00 ml Volumen gelöst sofort und nach 3 Stunden $\alpha_D^{20} = -12,53°$, $[\alpha]_D^{20} = -624,3°$.

9. *d*-Glucothiodiazolin

Aus einem Gemisch von 3,6 g *d*-Glucose in 6 ml Wasser mit 4,6 g 2-Phenylthiobenzhydrazid in 60 ml Alkohol, welches während 24 Stunden sein Drehungsvermögen im 1-dm Rohr von +2,84° nur bis auf +3,17° erhöhte, wurden im Laufe von weiteren 6 Tagen 1,15 g Kristallnadeln vom Schmp. 155°–157°[1], mit 6,82 % N und 7,81 % S und in Pyridin von $[\alpha]_D^{20} = +1212°$ ausgeschieden. Die Mutterlauge schied danach bei freiwilliger Eindunstung eine voluminöse Kristallmasse nebst Effloreszenzen ab; nach Erwärmen bis zum Lösen wurden beim Erkalten und nach Anreiben 2,8 g cremefarbene Nadeln oder nadelförmige Prismen vom Schmp. 145°–147°[1] ausgeschieden.

Für $C_{19}H_{22}O_5N_2S$ (390,45):

ber.:	C 58,44,	H 5,68,	N 7,18,	S 8,21 %,
gef.:	» 58,29,	» 5,80,	» 7,01,	» 8,07 ».

In Pyridin wie unten zeigte dieses Produkt $[\alpha]_D^{20} = +85,2°$; aus seiner Mutterlauge wurden bei der weiteren, freiwilligen Eindunstung noch 1,7 g kleine Prismen erhalten, welche bei 143°–146° schmolzen, 7,23 % N und 8,19 % S enthielten und $[\alpha]_D^{20} = -6,1°$ zeigten.

---

[1] Für das Glucothiodiazolin fand WUYTS (1) den Schmp. 147°–148°.

Bei einem neuen Versuche wurden aus einem Gemisch von 3,0 g *d*-Glucose in 10 ml 2-n. Salzsäure und 4,6 g 2-Phenylthiobenzhydrazid in 40 ml Alkohol im Laufe von 2 Tagen 2,0 g Kristallpulver vom Schmp. 147°–149° und $[\alpha]_D^{20} = +882°$ ausgeschieden. Bei der Umkristallisierung von 1,4 g davon aus 10 ml gew. Alkohol wurden 1,0 g schwach cremefarbene, nadelförmige Prismen vom Schmp. 154°–155° erhalten (Präp. 1). Die Mutterlauge des ersten Rohproduktes lieferte dann im Laufe von 2 Wochen noch 3,3 g Kristalle vom Schmp. 140°–142°, von denen 2,7 g bei der Umkristallisierung wie oben 2,2 g Nadeln vom Schmp. 143°–146° lieferten (Präp. 2); aus der neuen Mutterlauge wurden bei der freiwilligen Eindunstung zu 13 g Gewicht 1,5 g eines Produktes vom Schmp. 127°–130° und $[\alpha]_D^{20} = -170°$ gewonnen. Von diesem Produkte wurden 0,9 g mit 25 ml Äther behandelt, wobei ein ungelöster Rest von 0,6 g Gewicht und vom Schmp. ca. 135°–140° erhalten wurde (Präp. 3).

Die Analysen der verschiedenen Präparate ergaben:

Für $C_{19}H_{22}O_5N_2S$ (390,45):

ber.:	C 58,44,	H 5,68,	N 7,18,	S 8,21 %,
für Präp. 1 gef.:	» —	» —	» —	» 8,11 »
» » 2 »	» 58,03,	» 5,97,	» 7,21,	» 8,12 »
» » 3 »	» —	» —	» 7,14,	» 8,20 ».

Vom Präp. 1 zeigte eine Lösung von 0,2025 g in Pyridin auf 10,00 ml Volumen $\alpha_D^{20} = +24,96°$, $[\alpha]_D^{20} = +1233°$; Präp. 2 zeigte in der gleichen Weise $[\alpha]_D^{20} = +42,0°$ und Präp. 3 $[\alpha]_D^{20} = -287°$.

10. *d*-Mannothiodiazolin

Eine Lösung von 3,6 g *d*-Mannose in 10 ml 2-n. Salzsäure wurde am folgenden Tage mit 4,6 g 2-Phenylthiobenzhydrazid in 60 ml Alkohol versetzt, wonach das Gemisch nach *t* Minuten die folgenden Drehungen zeigte:

t:	3	10	20	40	80	150	320	610
α_D^{20}:	+1,22°	+1,12°	+0,96°	+0,68°	+0,17°	−0,53°	−1,88°	−3,63°.

Danach begann Kristallisation und nach 24 Stunden wurden 2,6 g mit 10 ml gew. Alkohol gewaschene, cremefarbene Nadeln vom Schmp. 201°–202°[1] erhalten (Präp. 1). Aus dem Filtrate wurden im Laufe von 2 Tagen 2,1 g Nadeln vom Schmp. 200°–202° ausgeschieden (Präp. 2). Nach einer Woche lieferte das neue Filtrat beim Absaugen 1,5 g Nadeln vom Schmp. 201°–202° (Präp. 3) und nach noch einer Woche 0,7 g vom Schmp. 197°–200° (Präp. 4). Das zuletzt erhaltene Filtrat wurde von 100 ml Wasser emulgiert und schied dann 0,5 g gelbliche Sternchen von schmalen Prismen vom Schmp. ca. 187°–192° aus (Präp. 5). Die Analysen der verschiedenen Präparate ergaben:

Für $C_{19}H_{22}O_5N_2S, H_2O = C_{19}H_{24}O_6N_2S$ (408,46):

ber.:	C 55,87,	H 5,92,	N 6,86,	S 7,85 %,
für Präp. 1 gef.:	» 55,74,	» 5,92,	» 6,85,	» 7,81 »
» » 2 »	» —	» —	» 6,85,	» — »
» » 3 »	» 55,86,	» 5,88,	» 6,90,	» 7,83 »
» » 4 »	» 55,61,	» 5,85,	» 6,88,	» 7,83 »
» » 5 »	» —	» —	» 7,29,	» 8,97 ».

---

[1] Wuyts fand für das Mannothiodiazolin den Schmp. 198° (1).

Das Präp. 1 verlor im Vakuum über Phosphorsäureanhydrid 4,36, ber. 4,41, % an Gewicht; von der getrockneten Probe zeigten 0,2334 g in Pyridin auf 10,04 ml Volumen gelöst sofort und nach einer Stunde $\alpha_{\mathrm{D}}^{20} = +18{,}18°$, $[\alpha]_{\mathrm{D}}^{20} = +782°$. In der gleichen Weise zeigten die analysierten, kristallwasserhaltigen Proben: Präp. 1: $[\alpha]_{\mathrm{D}}^{20} = +743°$, Präp. 2: $[\alpha]_{\mathrm{D}}^{20} = +69{,}6°$, Präp. 3: $[\alpha]_{\mathrm{D}}^{20} = +840°$, Präp. 4: $[\alpha]_{\mathrm{D}}^{20} = +879°$ und Präp. 5: $[\alpha]_{\mathrm{D}}^{20} = -234°$.

Bei der Umkristallisierung von 0,9 g vom Präp. 1 aus 25 ml gew. Alkohol wurden 0,8 g cremefarbene Nadeln vom Schmp. 201°–203° erhalten, welche wie oben $[\alpha]_{\mathrm{D}}^{20} = +883°$ zeigten und im Vakuum über Phosphorsäureanhydrid 4,31, ber. 4,41, % an Gewicht verloren, wonach die Probe bei der Analyse lieferte:

Für $C_{19}H_{22}O_5N_2S$ (390,45):

ber.: N 7,18, S 8,21 %; gef.: N 7,13, S 8,04 %.

Vom Präp. 3 ergaben 0,8 g bei der Umkristallisierung aus 20 ml gew. Alkohol 0,65 g schwach cremefarbene, nadelförmige Prismen vom Schmp. 198°–200° und wie oben von $[\alpha]_{\mathrm{D}}^{20} = +894°$. Dieses Produkt enthielt 4,46, ber. 4,41, % H_2O und in trockener Form 8,22, ber. 8,21 % S.

Als 0,5 g vom Präp. 2 in 10 Minuten auf 190° erhitzt und dann während der gleichen Zeit auf dieser Temperatur gehalten wurden, sinterte die Probe nur unbedeutend zusammen und zeigte danach $[\alpha]_{\mathrm{D}}^{20} = +856°$. Bei einem neuen Versuche wurde die Probe 20 Minuten bei 200°–203° gehalten, wobei eine gelbbraune Schmelze entstand, welche beim Erkalten glasig erstarrte und bei der Umkristallisierung aus 15 ml gew. Alkohol 0,35 g Nadeln vom Schmp. 200°–201° und $[\alpha]_{\mathrm{D}}^{20} = +877°$ lieferte.

11. *d*-Galaktothiodiazolin

Eine Lösung von 3,6 g *d*-Galaktose in 10 ml gelinde erwärmter 2-n. Salzsäure lieferte mit 4,6 g 2-Phenylthiobenzhydrazid in 60 ml Alkohol ein homogenes Gemisch, welches nach 4 Stunden $\alpha_{\mathrm{D}}^{20} = +23{,}37°$ zeigte. Bald danach begann Kristallisation und am folgenden Tage wurden beim Absaugen 3,0 g Sternchen aus kleinen, schwach cremefarbenen Nädelchen von Schmp. 179°–181°[1] erhalten.

Für $C_{19}H_{22}O_5N_2S$ (390,45):

ber.:	C 58,44,	H 5,68,	N 7,18,	S 8,21 %,
gef.:	» 58,22,	» 5,76,	» 7,19,	» 8,05 ».

Eine Lösung von 0,2402 g Sbst. in Pyridin auf 10,00 ml Volumen gelöst zeigte 0,5 $\alpha_{\mathrm{D}}^{20} = +13{,}32°$, $[\alpha]_{\mathrm{D}}^{20} = +1109°$.

Das Filtrat des obigen Produktes schied im Laufe von 2 Tagen 1,1 g Kristalle vom Schmp. 176°–180° aus, welche 6,96 % N und 7,92 % S enthielten und wie oben $[\alpha]_{\mathrm{D}}^{20} = -114{,}3°$ zeigten. Nach einer Woche wurden aus dem Filtrate noch 0,7 g Kristalle vom Schmp. 176°–180°, mit 7,01 % N und 8,01 % S und von $[\alpha]_{\mathrm{D}}^{20} = +598°$ erhalten. Die Mutterlauge ergab mit 100 ml Wasser eine Emulsion, welche 1,9 g eines Kristallkuchens vom Schmp. ca. 170°–175° und $[\alpha]_{\mathrm{D}}^{20} = +73{,}1°$ ausschied. Von diesem Produkte ergaben 0,8 g beim Fällen mit Wasser aus alkoholischer Lösung 0,6 g zu Sternchen vereinigte, nadelförmige Prismen, welche bei 175°–177° schmolzen und $[\alpha]_{\mathrm{D}}^{20} = +66{,}0°$ zeigten.

---

[1] Für das Galaktothiodiazolin hat WUYTS den Schmp. 178°–179° angegeben (1).

Bei einer Wiederholung des Versuches wurden zuerst 3,4 g eines Produktes vom Schmp. 180°–182° gewonnen, von dem 0,2206 g in Pyridin auf 10,00 ml Volumen gelöst sofort $\alpha_D^{20} = +23{,}35°$, $[\alpha]_D^{20} = +1059°$, und nach *t* Minuten die folgenden Drehungen zeigten:

t:	70	310	1440	1760	2880	4560
α_D^{20}:	+23,40°	+23,47°	+23,18°	+23,18°	+22,87°	+22,40°

Nach 2 Tagen wurden aus dem Filtrate des obigen Produktes 1,6 g Kristallpulver vom Schmp. 175°–178° erhalten; von diesem zeigten 0,2502 g in Pyridin wie oben gelöst sofort $\alpha_D^{20} = -3{,}20°$, $[\alpha]_D^{20} = -128{,}0°$, und danach:

t:	1530	3230	3960
α_D^{20}:	−3,10°	−2,92°	−2,88°

Als darauf 1,8 g *d*-Galaktose in 5 ml 2-n. Salzsäure wie oben mit 2,3 g 2-Phenylthiobenzhydrazid in 30 ml Alkohol versetzt wurden, schied das Gemisch im Laufe einer Woche 2,7 g Kristallpulver vom Schmp. 178°–180° aus, welches 6,89, ber. 7,18, % N enthielt, und von dem 0,2206 g in Pyridin wie oben sofort $\alpha_D^{20} = +14{,}55°$, $[\alpha]_D^{20} = +660°$, und danach die folgenden Drehungen zeigten:

t:	60	1690	4280	7170	12950
α_D^{20}:	+14,58°	+14,39°	+14,01°	+13,65°	+13,02°

Bei freiwilliger Eindunstung der Mutterlauge auf 4,4 g Gewicht wurden 0,8 g mit 10 ml Wasser und der gleichen Menge Alkohol gewaschenes Kristallpulver erhalten, welches bei ca. 165°–168° schmolz, 7,14, ber. 7,18, % N enthielt und in Pyridin wie üblich sofort $[\alpha]_D^{20} = +148°$ zeigte.

Bei einem ähnlichen Versuch, aber mit 4,6 g 2-Phenylthiobenzhydrazid, wurden zuerst 2,75 g Reaktionsprodukt vom Schmp. 179°–182° erhalten, welches in Pyridin wie oben sofort $[\alpha]_D^{20} = +723°$ zeigte.

Bei freiwilliger Eindunstung lieferte die Mutterlauge 7 g einer feuchten Kristallmasse, welche bei Behandlung mit 15 ml Alkohol, Absaugen und Waschen mit 5 ml Alkohol 0,4 g nicht einheitliches Kristallpulver lieferte, welches bei ca. 165°–169° schmolz und 7,73 % N enthielt. Das Filtrat lieferte bei freiwilliger Eindunstung 2,9 g Kristallkrusten, welche bei Behandlung mit 15 ml heissem Benzol einen ungelösten Rest von 0,5 g Gewicht, Schmp. 168°–170°, 7,00, ber. 7,18, % N und in Pyridin sofort $[\alpha]_D^{20} = +281°$ lieferte.

Als andererseits 3,6 g *d*-Galaktose in 5 ml gelinde erwärmter 2-n. Salzsäure mit 2,3 g 2-Phenylthiobenzhydrazid in 30 ml Alkohol umgesetzt wurden, lieferte das Gemisch beim Absaugen nach einer Woche 2,1 g mit 10 ml Alkohol gewaschenes Produkt vom Schmp. 164°–166° und mit 6,76, ber. 7,18, % N. Von diesem Produkte zeigten 0,2482 g in Pyridin wie gewöhnlich sofort $\alpha_D^{20} = +30{,}82°$, $[\alpha]_D^{20} = +1242°$, und danach:

t:	60	1390	4340	10130
α_D^{20}:	+30,80°	+30,30°	+29,54°	+27,85°

Beim Umkristallisieren von 1,6 g dieses Produktes aus 50 ml gew. Alkohol wurden 0,9 g nadelförmige Prismen erhalten, welche bei 180°–182° schmolzen, 7,10, ber. 7,18, % N enthielten und in Pyridin wie gewöhnlich sofort $[\alpha]_D^{20} = +1360°$ zeigten.

Das Filtrat des Rohproduktes lieferte bei freiwilliger Eindunstung 4,4 g einer sirupös gummiartigen Masse, welche mit 25 ml Wasser eine schwach trübe Lösung ergab.

Oxydationen des *d*-Galaktothiodiazolins mit Eisenchlorid, Pyridiniumtribromid oder Kaliumpersulfat lieferte keine einheitlichen Produkte.

12. Ketose und 2-Phenylthiobenzhydrazid

Die Darstellung eines Fructothiodiazolins gelang Wuyts (1) nicht, und auch unter den vom Verf. gewählten Versuchsbedingungen konnten keine Umsetzungsprodukte der Fructose oder der Sorbose mit dem 2-Phenylthiobenzhydrazid erhalten werden.

Bei dem Versuche mit der *d*-Fructose wurde eine Lösung von 3,6 g dieses Zuckers in 10 ml 2-n. Salzsäure am folgenden Tage mit 4,6 g 2-Phenylthiobenzhydrazid in 60 ml Alkohol versetzt, wobei ein homogenes Gemisch entstand, welches nach *St* Stunden die folgenden Drehungen zeigte:

St:	4/60	1	19,5	58,5	105	288	507
α_D^{20}:	− 3,45°	− 3,48°	− 3,78°	− 4,03°	− 4,58°	− 5,36°	− 5,58°

Das Gemisch wurde dann mit 100 ml Wasser versetzt, wobei eine Emulsion entstand, welche 3,9 g einer aus Öl erstarrten Kristallmasse vom Schmp. ca. 85°–90° ausschied. Nach der Umkristallisierung aus Benzol schmolz das Produkt bei 90°–91° und bestand somit aus regeneriertem 2-Phenylthiobenzhydrazid.

Ein in der gleichen Weise aus 1-Sorbose und 2-Phenylthiobenzhydrazid hergestelltes Gemisch zeigte:

St:	3/60	5	11	22	49	76	96	143	217
α_D^{20}:	− 2,28°	− 2,04°	− 1,76°	− 1,68°	− 1,78°	− 1,88°	− 1,98°	− 2,13°	− 2,23°

Beim Versetzen des Reaktionsgemisches mit Wasser wurde dann auch hier nur unverbrauchtes 2-Phenylthiobenzhydrazid, 3,8 g vom Schmp. 85°–88°, ausgefällt.

Åkarp, Schweden, Mai 1954.

LITERATURVERZEICHNIS:

1. M. H. Wuyts: C. R. *196*, 1678 (1933).
2. M. H. Wuyts u. R. Verstraeten: Bull. Soc. Chim. de Belg. *43*, 271 (1934).
3. — Acad. royale de Belg. Bull. de la classe des sciences, Sér. 5, *20*, 168 (1934), und Bull. Soc. Chim. de Belg. *44*, 323 (1935).
4. — Acad. royale de Belg. Bull. de la classe des sciences, Sér. 5, *21*, 415 (1935).
5. B. Holmberg: Arkiv f. kemi *4*, 33 (1952).
6. M. H. Wuyts u. Mlle A. Lacourt: Bull. Soc. Chim. de Belg. *42*, 1 (1933).
7. M. H. Wuyts u. H. Wachsmuth: J. de Pharm. et de Chim., Sér. 8, *22*, 289 (1935).
8. M. H. Wuyts u. Mlle A. Lacourt: Bull. Soc. Chim. de Belg.. *48*, 194 (1939).
9. — Acad. royale de Belg. Bull. de la classe des sciences, Sér. 5, *19*, 543 (1933).

Tryckt den 24 november 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 56

Vorgelegt am 2. Juni 1954

Über *l*-Fucose-thiobenzhydrazid

Von Bror Holmberg

Mit 1 Figur im Texte

Als Komplettierung zu einer früheren Mitteilung über Thiobenzhydrazide einfacher Zuckerarten (1) werden im Folgenden Darstellungen und Eigenschaften des *l*-Fucose-thiobenzhydrazids und seine Umsetzungen mit Eisenchlorid, *d,l*-α-Brompropionsäure und Formaldehyd beschrieben.

Bei der ersten Darstellung des *l*-Fucose-thiobenzhydrazids wurde eine Lösung von 0,8 g von *Pfanstiehl Chemical Co.*, Waukegan, Illinois, bezogener *l*-Fucose in 10 ml Wasser mit 1,0 g Thiobenzhydrazid in 10 ml Alkohol versetzt. Hierbei entstand ein homogenes Gemisch, welches nach einer halben Stunde Kriställchen abzuscheiden begann und bis zum folgenden Tage 1,3 g mit 10 ml Alkohol gewaschene, farblose, haarfeine Nädelchen vom Schmp. 192°–194° (Zersetzung) absetzte (Präp. 1). — Das gleiche Thiohydrazid wurde auch aus dem Blasentang, *Fucus vesiculosus* L., dargestellt[1], wobei zuerst 50 g lufttrockenes Mehl aus dieser Braunalge, 41 g Trockensubstanz entsprechend, 4 Stunden, gelegentlich umgerührt, mit 200 ml 1-n. Salzsäure auf dem Wasserbade erhitzt wurden. Nach Abnutschen des dabei ungelösten Materiales und Waschen desselben mit 200 ml heissem Wasser wurde das Filtrat mit Natronlauge neutralisiert, auf dem Wasserbade zu 100 g Gewicht eingedunstet und mit 100 ml Alkohol versetzt, wobei eine teigige Salzmasse ausgeschieden wurde. Diese Masse wurde abfiltriert und mit 25 ml 67 %igem Alkohol gewaschen, das Filtrat wie vorher zu 50 g Gewicht eingedunstet und dann nach dem Erkalten mit einer Lösung von 8 g Thiobenzhydrazid in 50 ml Alkohol versetzt. Während der Eindunstung und beim Zusatz der Thiohydrazidlösung noch ausgeschiedenes Salz wurde sogleich abfiltriert, wonach die Lösung bis zum folgenden Tage 5,1 g feine Nadeln vom Schmp. 186°–188° (Zersetzung) ausschied. Beim Umkristallisieren von 2,5 g dieses Produktes aus 200 ml gew. Alkohol wurden 1,9 g fast haarfeine Nadeln (Präp. 2) vom Schmp. 193°–195° (Zersetzung) erhalten.

Für $C_{13}H_{18}O_4N_2S$ (298,36):

ber.:	C 52,33,	H 6,08,	N 9,39,	S 10,75 %,
gef., Präp. 1:	» —	» —	» 9,41,	» 10,73 »,
» » 2:	» 52,37,	» 6,02,	» 9,40,	» 10,79 ».

---

[1] Bei der gleichen Behandlung ergab die Braunalge *Laminaria digitata* L. nur *d*-Glucose-thiobenzhydrazid, während dabei aus der Grünalge *Ulva lactuca* L., in welcher Röhrmann (2) ein Rhamnose lieferndes Methylpentosan gefunden hat, ein Gemisch aus gleichen Teilen der Thiobenzhydrazide der *l*-Rhamnose und der *d*-Glucose erhalten wurde.

Eine Lösung von 0,4961 g vom Präp. 1 in Pyridin auf 10,00 ml gelöst zeigte ohne Mutarotation $\alpha_D^{20} = +1{,}78^\circ$, $[\alpha]_D^{20} = +35{,}9^\circ$, während 0,5265 g vom Präp. 2 in der gleichen Weise $\alpha_D^{20} = +1{,}91^\circ$, $[\alpha]_D^{20} = +36{,}3^\circ$ und $[M]_D^{20} = +108{,}2^\circ$ ergaben. Für das Thiobenzhydrazid der *l*-Rhamnose wurde (1) unter denselben Verhältnissen $[\alpha]_D^{20} = +10{,}9^\circ$ gefunden.

Eine Lösung von 0,5050 g vom Präp. 2 in $1{,}69 + 0{,}17 = 1{,}86$ ml 1-n. Natronlauge, 5 ml Akohol und Wasser auf 10,00 ml Volumen zeigte *t* Minuten nach dem Zusatz der Lauge:

t:	10	15	30	120	720	1440
α_D^{20}:	$-0{,}97^\circ$	$-1{,}34^\circ$	$-1{,}58^\circ$	$-1{,}60^\circ$	$-1{,}64^\circ$	$-1{,}64^\circ$

$$[\alpha]_D^{20} = \rightarrow -32{,}5^\circ; \quad [M]_D^{20} = \rightarrow -96{,}9^\circ.$$

In der gleichen Weise zeigten 0,4358 g *l*-Rhamnose-thiobenzhydrazid in $1{,}46 + 0{,}15 = 1{,}51$ ml 1-n. Natronlauge usw. 10 Minuten nach dem Zusatz der Lauge $\alpha_D^{20} = -0{,}37^\circ$, 15 und 360 Minuten danach $\alpha_D^{20} = -0{,}42^\circ$, $[\alpha]_D^{20} = \rightarrow -9{,}6^\circ$.

Als eine Lösung von 1,5 g *l*-Fucose-thiobenzhydrazid in 50 ml warmem, 50%igem Alkohol mit 10 ml 2-m. *Eisenchlorid*-Lösung versetzt wurde, trat eine schnell vorübergehende Dunkelfärbung ein, und eine bräunlich gelbe Lösung entstand, welche beim Erkalten 0,9 g 5-*l*-Fucotetraoxyamyl-2-phenyl-1,3,4-thiodiazol als haarfeine Nadeln vom Schmp. ca. 220°–224° (Zersetzung) ausschied. Bei der Umkristallisierung von 1,9 g solcher Produkte aus 200 ml gew. Alkohol wurden 0,9 g Nadeln wie vorher und vom Schmp. 223°–225° (Zersetzung) erhalten.

Für $C_{13}H_{16}O_4N_2S$ (296,34):

ber.:	C 52,69,	H 5,44,	N 9,45,	S 10,82 %,
gef.:	» 52,72,	» 5,41,	» 9,49,	» 10,74 ».

Eine in der Wärme bereitete und nach dem Erkalten auf 20,04 ml Volumen verdünnte Lösung von 0,4020 g des Fuco-thiodiazols in Pyridin zeigte sogleich und nach 3 Stunden 2 $\alpha_D^{20} = -0{,}27^\circ$, $[\alpha]_D^{20} = -6{,}7^\circ$ und $[M]_D^{20} = -19{,}9^\circ$, während für das entsprechende Derivat der *l*-Rhamnose $[\alpha]_D^{20} = +26{,}6^\circ$ gefunden vurde (1).

Debye-Scherrer-Photogramme des *l*-Fucose-thiobenzhydrazids und des *l*-Fucotetraoxyamyl-phenylthiodiazols zusammen mit den früher von JOHANSSON (3) mitgeteilten Photogrammen der entsprechenden Derivate der *l*-Rhamnose zeigt das Bild an der S. 3.

Tabelle 1. *l*-Fucose-thiobenzhydrazid.

I	*d*	*I*	*d*	*I*	*d*	*I*	*d*
8	16,4	3	4,02	2	2,83	2	2,240
6	8,10	3	3,96	4	2,71	1	2,215
3	6,01	2	3,80	2	2,63	2	2,130
4	5,25	6	3,76	3	2,54	3	2,090
7	4,72	5	3,53	3	2,485	2	2,060
10	4,65	3	3,47	1	2,410	1	1,930
2	4,57	4	3,34	1	2,385	1	1,900
3	4,41	4	3,31	2	2,360	1	1,825
7	4,32	3	3,21	2	2,335	1	1,700
9	4,29	3	3,08	2	2,255	1	1,670
9	4,17	4	2,96				

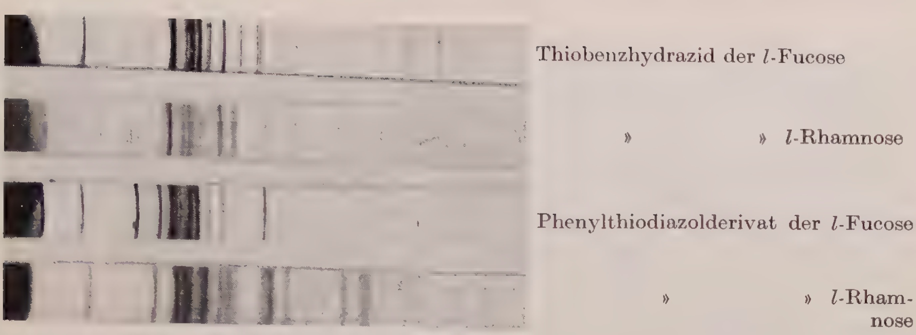

Thiobenzhydrazid der *l*-Fucose
» » *l*-Rhamnose
Phenylthiodiazolderivat der *l*-Fucose
» » *l*-Rhamnose

Fig. 1.

Die relativen Intensitäten, *I*, für die Linien der Fucosederivate und die diesen Linien entsprechenden Netzebenabstände *d* (in Å), sind in den Tabellen 1 und 2 angegeben.

Tabelle 2. 5-*l*-Fucotetraoxyamyl-2-phenyl-1,3,4-thiodiazol.

I	*d*	*I*	*d*	*I*	*d*	*I*	*d*
5	16,8	5	4,71	2	3,59	3	2,68
10	11,9	3	4,58	2	3,56	2	2,56
7	8,1	3	4,31	2	3,34	1	2,540
2	6,10	3	4,28	5	3,25	1	2,345
8	5,60	9	4,19	4	3,14	1	2,140
3	5,27	4	3,95	3	3,09	1	2,080
6	4,95	4	3,75	3	2,97	1	2,030

Die den stärksten Linien der gemessenen Photogramme der Fucose- und Rhamnosederivate entsprechenden Netzebenabstände zeigt Tabelle 3.

Tabelle 3.

	1	2	3
Thiobenzhydrazid der *l*-Fucose:	4,65	4,29	4,17
» » *l*-Rhamnose:	5,78	15,4	4,85
Phenylthiodiazolderiv. der *l*-Fucose:	11,9	4,19	5,60
» » *l*-Rhamnose:	4,45	4,08	15,2

Als 5 g *l*-Fucose-thiobenzhydrazid mit 17 ml 1-n. Natronlauge und 3 ml Alkohol geschüttelt wurden, entstand in einer Stunde eine homogene Lösung, welche mit 5 g mit 35 ml 1-n. Natronlauge neutralisierter *d,l-α-Brompropionsäure* versetzt wurde. Die am folgenden Tage unveränderte Lösung wurde dann von 10 ml 5-n. Salzsäure bald emulgiert und schied ein viskoses Öl ab. Die wässerige Schicht wurde mit 50 ml Äther extrahiert, wonach dieser mit 12 ml 1-n. Lauge neutralisiert wurde und dann bei der freiwilligen Eindunstung nur 0,2 g eines klebrigen Öles ergab. Das zuerst abgeschiedene Öl wurde in 100 ml Äther gelöst, wonach die Lösung von 7 ml 1-n. Lauge neutralisiert wurde und dann wie oben als Eindunstungsrest 1,1 g einer klebrigen Kristallmasse lieferte, welche sich nicht aus Alkohol umkristallisieren liess. Die neutralen, wässerigen Lösungen wurden vereinigt und das Gemisch mit 5 ml 5-n. Salzsäure versetzt, wobei noch etwas viskoses Öl abgeschieden wurde. Das Filtrat davon lieferte bei zweimaliger Extraktion mit 50 ml Äther Säuren 10,5 + 0,5 = 11 ml 1-n. Natronlauge entsprechend; die hierbei erhaltene, neutrale Wasserlösung ergab nach Zusatz von 1,8 g Kaliumxanthogenat usw. wie früher (1, S. 85–86) 0,9 g Dithiodilactylsäuren vom Schmp. ca. 105°–130°, Äquiv.-Gew. 106,8 und für 0,5603 g in Wasser auf 10,00 ml Volumen gelöst $\alpha_D^{20} = -0{,}27^\circ$, $[\alpha]_D^{20} = -4{,}8^\circ$, während in der gleichen Weise bei dem *l*-Rhamnose-thiobenzhydrazid (mit der umgekehrten Konfiguration des asymmetrischen, dem Carbonylkohlenstoff benachbarten Kohlenstoffatoms) ein Gemisch von Disulfidsäuren mit $[\alpha]_D^{20} = +0{,}3^\circ$ erhalten wurde.

Nach 7-stündigem Schütteln von 3,0 g *l*-Fucose-thiobenzhydrazid mit 1,0 g *Formalin* in 20 ml 0,5-n. Salzsäure wurden 2,1 g *l*-Fucose-methylenthiobenzhydrazid als weisses, mit 25 ml Wasser gewaschenes Kristallpulver vom Schmp. 132°–135° erhalten.

Für $C_{14}H_{18}O_4N_2S$ (310,37):

ber.:	C 54,17,	H 5,85,	N 9,03,	S 10,33 %,
gef.:	» 54,17,	» 5,82,	» 9,06,	« 10,38 ».

Eine Lösung von 0,5038 g des Produktes in Pyridin auf 10,00 ml Volumen gelöst zeigte nach *t* Minuten:

t:	12	30	60	120	240	420	720
$0{,}5\ \alpha_D^{20}$:	$-1{,}79^\circ$	$-1{,}77^\circ$	$-1{,}74^\circ$	$-1{,}62^\circ$	$-1{,}57^\circ$	$-1{,}54^\circ$	$-1{,}54^\circ$

$[\alpha]_D^{20} = -72{,}2^\circ$[1] $\rightarrow -61{,}1^\circ$; $[M]_D^{20} = -224^\circ \rightarrow -190^\circ$.

Für das *l*-Rhamnose-methylenthiobenzhydrazid in Pyridin wurde $[\alpha]_D^{20} = -162{,}7^\circ$ (ohne Mutarotation) gefunden.

Als bei einem neuen Versuch 2,7 g *l*-Fucose-thiobenzhydrazid 6 Stunden mit 1,0 g Formalin und 20 ml 0,5-n. Salzsäure geschüttelt wurden, verblieben 0,35 g davon vom Schmp. 189°–191° ungelöst. Das Filtrat davon wurde von 5 g Thioglykolsäure schnell getrübt und schied bis zum folgenden Tage 2,0 g Kristallpulver vom Schmp. 143°–145° und der Zusammensetzung einer Methylenthiobenzhydrazino-fucose-thioglykolsäure ab.

Für $C_{16}H_{22}O_6N_2S_2$ (402,48):

ber.:	C 47,74,	H 5,51,	N 6,96,	S 15,93 %,
gef.:	» 47,63,	» 5,50,	» 6,99,	» 15,81 ».

Eine Lösung von 0,2207 g des Produktes in Pyridin auf 10,00 ml zeigte:

t:	10	20	100	200	720	1460	2170	4350	6510	7300
α_D^{20}:	$-10{,}86^\circ$	$-10{,}72^\circ$	$-9{,}85^\circ$	$-8{,}87^\circ$	$-5{,}15^\circ$	$-1{,}97^\circ$	$-0{,}64^\circ$	$+0{,}51^\circ$	$+0{,}73^\circ$	$+0{,}73^\circ$
$C_M^{20} \times 10^4$:	—	—	10,1	9,9	9,6	10,0	9,9	—	—	—

$[\alpha]_D^{20} = -500^\circ \rightarrow +33{,}1^\circ$; $[M]_D^{20} = -2010^\circ \rightarrow +133^\circ$. C_M^{20}[2] $= 9{,}9 \times 10^{-4}$.

---

[1] Hier wie im Folgenden ist der Null-Wert geschätzt worden.
[2] Geschwindigkeitskonstante der Mutarotation (Zeit in Minuten).

Beim Umkristallisieren von 2,4 g derartiger Produkte aus 30 ml gew. Alkohol wurden 1,1 g mit 10 ml desselben Alkohols und der gleichen Menge Äther gewaschenes, feinkörniges Kristallpulver erhalten, welches bei 150°–152° schmolz und 15,86, ber. 15,94, % S enthielt. Eine Lösung von 0,4139 g Sbst. in 10 ml gew. Alkohol ergab mit 10,30 ml 0,1000-n. Natronlauge einen etwas unscharfen Phenolphthalein-Umschlag und verbrauchte dann mit schwach gleitendem Umschlag 10,26 ml 0,1000-n. J-Lösung, Äquiv.-Gew. ber. 402,5, gef. 401,8 bzw. 403,4. — Eine Lösung von 0,2110 g in Pyridin auf 10,00 ml zeigte:

t:	10	300	1440	2880	4320
α_D^{20}:	+0,45°	+0,55°	+0,65°	+0,70°	+0,70°

$[\alpha]_D^{20} = +21° \rightarrow +33,2°$; $[M]_D^{20} = +86° \rightarrow +134°$.

Als andererseits 1,7 g *l*-Fucose-methylenthiobenzhydrazid mit 2 g Thioglykolsäure in 10 ml 1-n. Salzsäure zusammengerieben wurden, entstand ein dünner Brei, welcher am folgenden Tage mit 20 ml Wasser versetzt wurde, wonach beim Absaugen und Waschen mit 10 ml Wasser 1,7 g Thioglykolsäure-Produkt als undeutlich kristallinisches Pulver vom Schmp. 136°–138° erhalten wurden. Dieses Produkt enthielt 16,35, ber. 15,93, % S und 0,2022 g davon, in Pyridin auf 10,00 ml Volumen gelöst, zeigten:

t:	10	15	120	240	420	720	1480	2920	4360	5800	7240	8680
α_D^{20}:	−8,82°	−8,77°	−7,98°	−7,10°	−5,97°	−4,40°	−1,97°	−0,12°	+0,37°	+0,52°	+0,63°	+0,63°
$C_M^{20} \times 10^4$:	—	—	8,5	8,7	8,8	8,9	8,8	8,7	8,3	—	—	—

$[\alpha]_D^{20} = -442° \rightarrow +31,2°$; $[M]_D^{20} = -1780° \rightarrow +125,5°$. $C_M^{20} = 8,7 \times 10^{-4}$.

Beim Umkristallisieren von 1,2 g des obigen Produktes aus 10 ml gew. Alkohol wurden 0,6 g flache Prismen vom Schmp. 149°–152° und mit 15,87, ber. 15,93, % S erhalten. Hiervon zeigten 0,2109 g in Pyridin auf 10,00 ml gelöst:

t:	10	20	120	240	420	720	1320	2880	5760	7200
α_D^{20}:	−1,22°	−1,21°	−1,05°	−0,89°	−0,69°	−0,37°	+0,03°	+0,52°	+0,62°	+0,62°
$C_M^{20} \times 10^4$:	—	—	8,8	8,6	8,3	8,5	8,7	—	—	—

$[\alpha]_D^{20} = -58,3° \rightarrow +29,4°$; $[M]_D^{20} = -235° \rightarrow +118°$. $C_M^{20} = 8,6 \times 10^{-4}$.

Als ein Gemisch aus 1,5 g *l*-Fucose-thiobenzhydrazid, 1,6 g *d*-Glucose-thiobenzhydrazid, 19 ml Wasser, 20 ml Alkohol und 1,0 g Formalin geschüttelt wurde, entstand in 1½ Stunden eine klare Lösung, welche mit *d*-Glucose-methylenthiobenzhydrazid geimpft wurde und dann im Laufe von 3 Tagen 0,5 g mit 5 ml 50%igem Alkohol gewaschene, haarfeine Nadeln lieferte, vom Schmp. 184°–186° und für 0,4449 g in Pyridin auf 10,00 ml Volumen gelöst $\alpha_D^{20} = -7,24°$, $[\alpha]_D^{20} = -162,7°$, während früher (1) für das *d*-Glucose-methylenthiobenzhydrazid der Schmp. 188°–189° und (in Pyridin) $[\alpha]_D^{20} = -165°$ gefunden wurden. Das Filtrat schied dann allmählich noch 0,7 g desselben Produktes vom Schmp. 183°–185°, mit 51,57, ber. 51,52, % C und 5,53, ber. 5,56, % H und (in Pyridin) von $[\alpha]_D^{20} = -162,9°$ aus. Das Filtrat davon lieferte mit 4 g Thioglykolsäure und 5 ml 2-n. Salzsäure ein homogenes Gemisch, welches bald Kristalle abzuscheiden begann und bis zum folgenden Tage 0,8 g unreine Methylenthiobenzhydrazino-fucose-thioglykolsäure als feinkristallinisches Pulver ausschied. Das Produkt schmolz bei 136°–138°, enthielt 6,53, ber. 6,96, % N und 15,02, ber. 15,93, % S; 0,2056 g davon, in Pyridin auf 10,00 ml Volumen gelöst, zeigten:

t:	10	15	20	100	300	700	1470	2880	4320	5760	7200	8640	10020
α_D^{20}:	$-9,76°$	$-9,72°$	$-9,66°$	$-9,08°$	$-7,74°$	$-5,53°$	$-2,72°$	$-0,57°$	$+0,33°$	$+0,53°$	$+0,63°$	$+0,68°$	$+0,68°$
$C_M^{20} \times 10^4$:	—	—	—	7,5	7,4	7,5	7,7	7,4	7,9	—	—	—	—

$[\alpha]_D^{20} = -480° \rightarrow +33,1°$. $C_M^{20} = 7,6 \times 10^{-4}$.

Diese Uuntersuchung wurde in der damaligen org.-chem. Abteilung des Nobelinstitutes der Kgl. Akademie der Wissenschaften, Stockholm, ausgeführt. Für die Analysen hat der Verf. Frau Majbritt Vestergaard, Stockholm, für die Röntgenphotogramme dem Hrn. Privatdozent Dr. A. Magnéli, Uppsala, und für die zugehörigen Tabellen dem Hrn. Dr. Axel Johansson, Kgl. Technische Hochschule, Stockholm, bestens zu danken

Åkarp, Schweden, Januar 1954.

LITERATURVERZEICHNIS

[1] B. Holmberg: Arkiv f. Kemi *4*, 33 (1952).
[2] Röhrmann: Festschr. f. Salkowski (1904), zitiert nach Czapeck, Bioch. d. Pflanzen, 2. Aufl., S. 641 (1913).
[3] A. Johansson: Arkiv f. kemi *4*, 17 (1952).

Tryckt den 29 november 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 57

Communicated 8 September 1954 by ARNE TISELIUS

Charcoal chromatography with a step-graded adsorption column

By J. PORATH

With 2 figures in the text

Pretreatment of charcoal by saturators has been proposed and discussed by TISELIUS and his collaborators (1–4). The pretreatment can be performed by a "flowing" or a "stationary" saturator. In the first case the saturator is included in all solvents used and will serve the additional purpose of an eluting agent. Accumulated data support the view that charcoal chromatography of strongly adsorbable peptides can only be performed successfully on charcoal saturated under strictly controlled conditions (5). Presumably, a relatively simple mixture can be resolved by repeated chromatography on a number of columns containing charcoal through which the appropriate saturators are flowing. However, for a complex mixture, this procedure may be very laborious.

When stationary saturation is used, no saturator is included in the solvents. This pretreatment consists of adsorbing a substance which is so strongly held to the charcoal that it will not be eluted or displaced under the condition applied for the chromatography of the mixture to be fractionated.

The following method is proposed for group separation of substances which are more or less strongly adsorbed on charcoal pretreated with a stationary saturator. A column is constructed of sections containing charcoal of different degrees of saturation in a way such that discontinuous incremental adsorption is established. In such a step-graded column where the adsorption power varies by discrete stages, the charcoal is more strongly saturated at the top than at the bottom and consequently a substance will move through regions of saturator concentration stronger than that of the critical saturation point (5) and will be adsorbed in a region from which it can easily be eluted with good yield.

As will be shown below, a step-graded column may yield an efficient group separation in a single chromatogram. The sectional column constructed by HAGDAHL may conveniently be adapted for that purpose (6).

For satisfactory adsorption it is essential that the saturator is adsorbed uniformly on the charcoal and this was achieved by the following simple procedure.

A mixture of 25 g of Carbo Active (Merck-Darmstadt) and 75 g of Celite was suspended in 300 ml of boiling ethanol and placed on a boiling water bath. A weighed amount of stearic acid, dissolved in 250 ml of hot alcohol was added slowly to the suspension, heated until the total volume decreased to 300 ml and the suspension

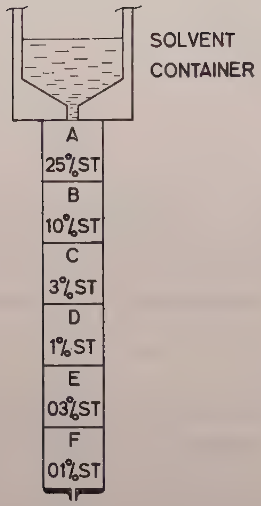


Fig. 1.

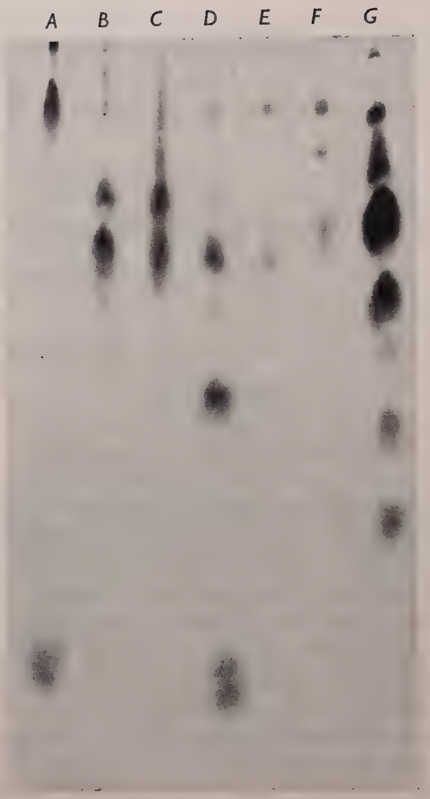


Fig. 2.

Fig. 1. Schematic drawing illustrating the principle of a step-graded column used for fractionation of pea root exudate. All sections are of the same size (1570 mm³) and filled with charcoal-Celite mixture treated with stearic acid in concentrations varying from 25 per cent at the top to 0.1 per cent at the bottom of the column.

Fig. 2. Paper chromatogram of fractions of pea root exudate obtained from a step-graded column. The chromatogram was run with butanol-acetic acid-water (4:1:5) on Whatman No. 1 paper for 48 hours. For details see text.

allowed to cool to room temperature. After introduction of the stearic acid solution continuous stirring must be maintained. The ethanol was removed by filtering under suction. The charcoal-celite mixture was thoroughly washed with three 200 ml portions of 50 per cent *n*-propanol and finally with at least one liter of distilled water in small portions. In order to determine the amount of stearic acid adsorbed all alcohol solutions were evaporated and the recovered stearic acid was weighed. This decreased rapidly and was insignificant in the last washing. The maximum amount of stearic acid which could be adsorbed on charcoal was found to be about 30 per cent of the charcoal on a weight basis.

To illustrate the results and operations of step-graded columns the fractionation of pea root exudate is considered. Fig. 1 illustrates the arrangement of the sections of the column. Concentrated pea root exudate (1.2 ml) containing 20 mg dry material was slowly pressed into the column. The chromatogram was developed by washing with 15 ml of distilled water (6 ml/hour). The column was dismantled and the material in the different sections eluted with 50 per cent *n*-propanol. The eluates were evaporated and investigated by paper chromatography and paper electrophoresis. The paper chromatogram obtained when the system butanol-acetic acid-water (4:1:5) was used is shown in Fig. 2. Fraction (*A*) was obtained from the upper section *A*, fraction (*B*) from section *B*, etc. The material in fraction (*G*) had passed through the column without being adsorbed. As far as the ninhydrin-positive material is concerned, peptides and substances of aromatic character were adsorbed principally in the upper sections, while the eluates of the lower sections and fraction (*G*) consisted mainly of amino acids. Most of the carbohydrates were recovered in fraction (*G*) which also contained all inorganic salts. The distribution of material absorbing ultra-violet light at 235 mμ and 265 mμ also shows that an efficient group separation with respect to purine and pyrimidine compounds was achieved. A more detailed description of fractionation of pea root exudate will be published later.

The advantages of the proposed procedure can be summarised as follows: (1) Charcoal chromatography of a mixture consisting of components of widely differing adsorbability can be performed with high total yield (90–100 per cent recovery) in one single chromatogram. (2) An efficient group separation can be achieved. (3) The material obtained in the upper section in the experiment described above can be distributed over a whole column, and thus fractionated by superimposing a flowing saturator on the stationary saturator of the graded adsorption column. (4) Substances which are adsorbed on the column are freed of inorganic salts. (5) The use of modified charcoal makes it possible to perform the operation under mild conditions. (6) There is no detectable contamination from the adsorbent. (7) The column can be regenerated after use by washing with water. A regenerated column will maintain its original properties in so far as the results of paper chromatography of corresponding fractions can be used as a criterion.

The author wishes to express his gratitude to Professor A. Tiselius for valuable discussions and to Dr N. Fries and Mr B. Forsman who kindly supplied the author with the pea root exudate sample. He is also indebted to Mr S. Jerstedt for skilful technical assistance.

This work is part of a research program carried out with financial aid from the Rockefeller and Wallenberg Foundations and from the Swedish Natural Science Research Council.

Institute of Biochemistry, University of Uppsala, Uppsala, Sweden.

REFERENCES

1. Tiselius, A. and Hahn, L., Kolloid-Z. *105*, 177 (1943).
2. Synge, R. L. M. and Tiselius, A., Acta Chem. Scand. *3*, 231 (1949).
3. Hagdahl, L., Williams, R. J. P., and Tiselius, A., Arkiv Kemi *4* (1952) No. 10.
4. Williams, R. J. P., Hagdahl, L., and Tiselius, A., Arkiv Kemi *7* (1954) No. 1.
5. Porath, J. and Li, C. H., Biochim. Biophys. Acta *13*, 268 (1954).
6. Hagdahl, L., Acta Chem. Scand. *2*, 574 (1948).

Tryckt den 29 november 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 58

Communicated 22 September 1954 by EINAR HAMMARSTEN and ERIK JORPES

Studies on enterokinase

I. The purification and general properties

By IKUO YAMASHINA*

With 1 figure in the text

In 1899, SCHEPOVALNIKOFF (1), working in PAVLOFF's laboratory, discovered that intestinal secretion activated the proteolytic activity of the pancreatic juice which is secreted in an inactive state as previously shown by KÜHNE (2) and HEIDENHAIN (3). Pancreatic juice to which duodenal secretion was added to 1–10 % hydrolyzed fibrin three or four times quicker than did the pancreatic juice itself. Duodenal fluid contents had no proteolytic activity. Mainly because of the heat sensitivity of the activator, PAVLOFF presumed that the activating power must be due to an enzyme which might be called "an enzyme of enzymes" and named it enterokinase (4). In his Nobel Lecture in Stockholm (*Les Prix Nobel*, 1904) he laid stress on the fact that the proteolytic enzymes of the pancreas, which could turn out to be dangerous for their fellow enzymes, the amylase and the lipase, are delivered from the gland in an inactive state.

Afterwards some of the many authors working in this field denied the catalytic action of the enterokinase, and others supported the concept of the Russian workers. As could be expected, this activator of the proteolytic activity exerted an attraction on the people working with the proteolytic enzymes. Thus WALDSCHMIDT-LEITZ (5, 6), a pupil of WILLSTÄTTER, and KUNITZ (7–10), the coworker of NORTHROP, paid particular attention to this substance. The history was reviewed by KUNITZ (7) in 1939. In the same year KUNITZ (8) established that enterokinase is an enzyme which catalyzes the transformation of trypsinogen to trypsin. At the same time, he purified the enterokinase to a certain extent and suggested that it might be a glycoprotein (9).

In fact, he obtained an enterokinase preparation having 40 enterokinase units per mg dry weight by ammounium sulfate fractionation under pH control. The analyses showed that it contained protein, a high content of carbohydrate, 10 % or less, and of aminosugar, 10 % or less. Taking into account the great success achieved by the NORTHROP group in crystallizing proteolytic enzymes by means of similar purification procedures, this preparation must have been regarded as nearly pure.

---

* Permanent address: Chemistry Department, Faculty of Science, Nagoya University, Nagoya, Japan. Present address: Chemistry Department II, Karolinska Institutet, Stockholm, Sweden.

Since I got an opportunity to work in this field, I decided to try to submit the enterokinase preparation of KUNITZ to further purification. About 15 times stronger preparations were in fact obtained. The properties of these preparations are shortly described.

Experimental

Estimation of enterokinase activity

The activity of enterokinase was measured according to the standardization method of KUNITZ (8) using trypsinogen as the substrate which was crystallized once and purified further with the aid of trichloroacetic acid as described by NORTHROP and KUNITZ (10). During the purification experiments, however, glycerol extract of acetone dried beef pancreas was used routinely as the substrate, whereby the tedious work in preparing quantities of crystalline trypsinogen could be avoided.

The activity of the preparations was expressed in terms of enterokinase units per protein mg as determined by means of LOWRY's (11) colorimetric method.

Preparation of the starting material

The enterokinase was prepared from frozen swine duodenal fluid contents according to the description of KUNITZ (9). His procedure was followed to step 10 (the second ammonium sulfate fractionation), followed by dialysis and lyophilization. Starting from 2.5 liters of duodenal fluid contents, 5 g of freeze-dried material was usually obtained. Despite a careful attempt to follow KUNIT's description, the preparation showed about 20 units per mg dried substance instead of 40.

Properties of the starting material

Prior to the purification, some properties of the starting material were investigated.

(1) *Stability of enterokinase at various pH values and temperatures.*—The behavior of enterokinase in this respect has never been investigated except for in the classical work of WALDSCHMIDT-LEITZ (5) who used a very crude preparation.

For the pH values 1, 2 and 3, diluted HCl was used, for pH 4 and 5, acetate buffer, for pH 8, 9, 10 and 11, ammonium buffer, and for pH 12 and 13, diluted NaOH. Each reaction mixture was incubated at room temperature (20° C) for 24 hrs., afterwards diluted, adjusted to pH 5.6 and assayed. The enterokinase proved to be completely stable under these conditions between pH 4 and 11, the activity being only slightly reduced at pH 3.

The influence of the temperature was studied at pH 5.6 in acetate buffer. The activity was completely lost after 5 min. at 100° C, 10 min at 80°, and 15 min. at 60°. However, at temperatures below 38°, no inactivation took place at least during 24 hrs. These data are not markedly different from those of Waldschmidt-Leitz.

(2) *Paper electrophoresis.*—The starting material was analyzed by means of paper electrophoresis at various pH values according to the directions of TISELIUS and KUNKEL (12). At pH 8.8 (veronal buffer, ionic strength 0.05), at least four components, migrating to the anode, could be demonstrated.

Purification

The following fractionation procedure was adopted after repeated trials.

(1) *Ethanol fractionation at pH 4.7.*—The starting material was dissolved to a concentration of 2.5 % in 0.01 M ammonium acetate-acetic acid buffer, pH 4.7, and cooled to 0° C in a cold bath with a constant temperature. Pre-cooled ethanol (99.5 %) was added dropwise with mechanical stirring. After removal through centrifugation at 0° C of the first precipitate obtained between 0 and 20 %[1] ethanol, ethanol was added to the supernatant to a concentration of 30 %. The first precipitate was dissolved in the same buffer as before and submitted to a renewed fractionation. The precipitates obtained between 20 and 30 % ethanol concentration were mixed, centrifuged at 0° C and lyophilized. This fraction, called Fraction 2, had an activity of 80 units per mg protein and the yield was about 70 %.

(2) *Acetone fractionation at pH 5.5 in the presence of* Zn^{+2}.—Fraction 2 was dissolved in pH 6.0 zinc-acetate-acetic acid buffer (Zn^{+2} conc. = 0.01 M) to 1 %, giving a final pH of 5.5, and cooled to 0° C. Pre-cooled 50 % acetone (50 % of acetone + 50 % of the buffer used as the solvent) was added with mechanical stirring. A bulky precipitate obtained between 0 and 20 % acetone concentration was centrifuged off, followed by the addition of 50 % acetone to the clear supernatant and the fraction obtained between 20–40 % acetone concentration was collected by centrifugation at 0° C.

This fraction, called Fraction 3, had an activity of about 200 units per mg protein and the yield, as referred to the starting material, was about 40 %.

(3) *Ethanol fractionation in the presence of* Ba^{+2}.—Fraction 3 was without previous lyophilization dissolved in 0.01 M ammonium acetate-acetic acid buffer containing 0.01 M barium acetate (pH 8.0) to a concentration of 1 % giving a final pH of 7.3. The fractionation by 50 % ethanol, which is composed of 50 % of ethanol (99.5 %) and 50 % of the buffer used as the solvent, was carried out at 0° C. The precipitate obtained at 20–30 % ethanol concentration was collected and submitted to a renewed fractionation procedure. Finally, the fractions obtained at 30–40 % ethanol concentration were collected and lyophilized. This preparation, called Fraction 4, had an activity of about 630 units per mg air-dried substance and the yield, as referred to the starting material, was about 15 %.

Further experiments with Fraction 4

(1) *Paper electrophoresis.*—Fraction 4, the best preparation of enterokinase so far obtained, was subjected to paper electrophoresis at various pH values from 3.8 to 8.8. As the buffer solution, a wide range buffer of veronal-acetic acid-sodium acetate was used.

At pH values from 6.2 to 8.8, there was one distinct spot followed closely by a weaker spot on its cathodic side. These findings suggest that there are at least two components closely related with respect to mobility in the aforementioned pH region. Indeed, at pH values below 6, two distinct spots were observed. The figures for the mobilities, expressed in terms of the distances from the starting point and corrected for the electro-osmotic flow, indicated, when plotted against pH values, that there were two components in Fraction 4, with the isoelectric points at about pH 4 and pH 5 respectively.

---

[1] The numbers are expressed in terms of volume percentage.

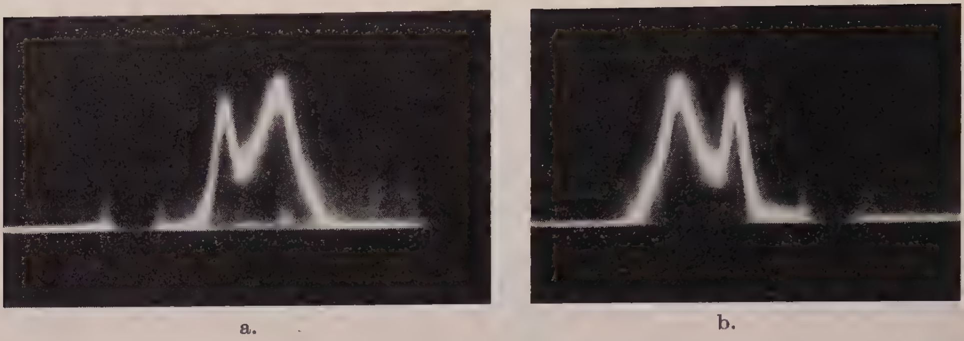

a. b.

Fig. 1. Electrophoretic patterns of Fraction 4 of enterokinase.—Acetate buffer: pH 4.4; ionic strength, 0.05 (containing sodium chloride—ionic strength, 0.15); temperature 1° C; current 32 mA; protein concentration, 1 %; time, 180 min. (*a*) Descending boundary; (*b*) ascending boundary.

Table 1. Analysis of the electrophoretically homogeneous substances. (All figures expressed in terms of μg in 0.5 ml of the solution.)

	Anionic component	Cationic component
Protein	550	170
Carbohydrate	56	0
Aminosugar	46	—

(2) *Electrophoresis with the Tiselius apparatus.*—The next step was to submit Fraction 4 to electrophoresis in free solution in the Tiselius-Svensson apparatus. A 1 % of solution of the preparation was dialyzed overnight against acetate buffer (ionic strength, 0.05 and pH 4.4) which contained sodium chloride (ionic strength, 0.15).

In accordance with the results of paper electrophoreses, there were observed two components at pH 4.4 as shown in Fig. 1.

(3) *Chemical analyses.*—Since each component could fortunately be taken out from the electrophoresis cell, even though in a small amount of solution, semi-quantitative analyses of each component were performed (Table 1). The protein content was determined according to the method of LOWRY (11), taking the starting material of enterokinase as the reference substance and carbohydrate according to the method of Dische as modified by GURIN and HOOD (13), with glucose as standard. The aminosugar determination was carried out according to the directions of GARDELL (14) after the hydrolysis by 6 N hydrochloric acid at 100° C for 8 hrs.

As regards to the activity, the negatively charged component had all of the activity of Fraction 4.

Discussion

Taking into consideration the results of the paper electrophoresis, the component having the isoelectric point at pH about 4 is enterokinase. Judging from the analytical figures and the electrophoretic behaviour it is quite possible that the enterokinase is a mucoprotein.

Summary

Enterokinase was prepared according to the procedure of KUNITZ from swine duodenal fluid contents and used as the starting material for purification experiments.

The influences of pH and temperature on the stability of enterokinase were studied. The activity was stable over a fairly wide pH region (4–11) but rather sensitive to heating, being inactivated completely after 15 min. even at 60° C.

Paper electrophoresis of the starting material showed that it was fairly crude. The purification was performed as follows: (1) Ethanol fractionation at pH 4.7, (2) Acetone fractionation at pH 5.5 in the presence of zinc, (3) Ethanol fractionation at pH 7.3–8.0 in the presence of barium. All fractionations were carried out at 0° C.

The purest preparation was 15 times stronger than the best one of KUNITZ's preparations. The electrophoresis on paper and of the free solution showed that this preparation contained two components having an isoelectric point at pH 4 and 5 respectively. The former had all the activity. It contained about 10 % carbohydrate and about the same amount of aminosugar.

The author's thanks are due to Professor ERIK JORPES and First Assistant VICTOR MUTT for their help during the performance of the work. It was made possible through research grants to professor Jorpes from the Swedish manufacturer of insulin, Vitrum Ltd., *Knut och Alice Wallenbergs Stiftelse*, and the Swedish Cancer Society.

Chemistry Department II, Karolinska Institutet, Stockholm, Sweden.

REFERENCES

1. SCHEPOVALNIKOFF, N. P., Maly's Jahresber. *29*, 378 (1900).
2. KÜHNE, W., Arch. Path. Anat. *39*, 130 (1867).
3. HEIDENHAIN, R., Arch. ges. Phys. *10*, 557 (1874).
4. PAVLOFF, I. P., Sämtliche Werke I–V, Akademie-Verlag, Berlin, 1953.
5. WALDSCHMIDT-LEITZ, E., Z. physiol. Chem. *132*, 181 (1923–24).
6. —— ibid. *142*, 217 (1924–25).
7. KUNITZ, M., Enzymologia *7*, 1, 1939.
8. —— J. Gen. Physiol. *22*, 429 (1939).
9. —— ibid. *22*, 447 (1939).
10. KUNITZ, M. and NORTHROP, J. H., J. Gen. Physiol. *19*, 991 (1936).
11. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J., J. Biol. Chem. *193*, 265 (1952).
12. KUNKEL, H. G. and TISELIUS, A., J. Gen. Physiol. *35*, 89 (1951).
13. GURIN, S. and HOOD, D. B., J. Biol. Chem. *131*, 211 (1939).
14. GARDELL, S., Acta Chem. Scand. *7*, 207 (1953).

Tryckt den 29 november 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 59

Vorgelegt am 26. Mai 1954

Untersuchungen am Yoshida-Ascites-Sarkom der Ratte. III[1]

Von HANS VON EULER und HANS HASSELQUIST

Die im Folgenden mitzuteilenden Beobachtungen und Versuche beziehen sich auf Ratten, in welchen wir das von T. YOSHIDA[2] 1944 entdeckte und in seinem Institut propagierte Ascites-Sarkom fortpflanzen. Mit diesen Ascites-Sarkomzellen, die sich in der Bauchhöhle teils als einzelne Zellen vermehren, teils an die Organe infiltrierend anlagern, injizieren wir teils unsere norwegischen Albinoratten, teils Ratten aus verschiedenen, hier ausgeführten Kreuzungen von ,,Gelb BD I'', die bei jedem Versuch näher angegeben sind.

Das Auftreten von Gallenfarbstoffen im Yoshida-Ascites

In etwa 1/3 aller mit Yoshida-Ascites-Sarkomzellen injizierten Ratten war der nach 3–5 Tagen auftretende Ascites intensiv gelb gefärbt. Der daraus mit Chloroform oder Benzol erhaltene gelbe Extrakt erwies sich durch die bekannten Reaktionen (siehe unten) als überwiegend *Bilirubin*, begleitet von wenigstens *einem* weiteren Gallenfarbstoff.

Bilirubin (synthetisiert von HANS FISCHER 1942) entsteht bekanntlich im Tierkörper aus Hämoglobin, wobei die (zwischen den die Vinylgruppen tragenden Pyrrolkernen befindliche) Methingruppe aus dem Porphyrinringsystem oxydativ entfernt wird und eine offene Pyrrolringkette entsteht. Vermutlich setzt diese Umwandlung schon ein, während das Hämin noch an Fe und Globin gebunden ist.

Über die Lokalisation des Überganges Hämoglobin-Bilirubin und über die dabei beteiligten Enzyme finden sich in der Literatur nur wenig Angaben.

Im normalen Rattenblut fanden wir in 3 Versuchen den mittleren Bilirubin-Gehalt $< 12\ \gamma$ Bilirubin/ml Blutserum.

Versuch zur Isolierung und Identifizierung des gelben Gallenfarbstoffes der Ascitesflüssigkeit

50 ml deutlich gelbe Ascites-Flüssigkeit (gesammelt von mehreren Tieren) wurde durch Centrifugieren von den Ascites-Sarkomzellen befreit, und die klare gelbe Lösung wurde mehrmals mit Benzol extrahiert bis die Benzolschicht sich nicht

---

[1] Frühere Mitt. dieser Serie: Dieses Arkiv *7*, 29 und *7*, 43 27. Januar und 14. April 1954.

[2] T. YOSHIDA, Y. MUTA u. Z. SASAKI, Proc. Imp. Acad. Tokyo *2* P, 611 (1944); T. YOSHIDA, J. National Cancer Inst. *12*, 947 (1952). — Siehe auch S. MAKINO und NAKAHARA, Z. Krebsforsch. *59*, 298 (1953). — Die Entwicklung des Yoshida-Sarkomes aus 1 Zelle gelang ISHIBUSHI, Gann, *41*, 1 (1953) und 236 (1950). — *42*, 233 (1951).

mehr färbte. Die vereinigten Benzolextrakte wurden mit wasserfreiem Natriumsulfat getrocknet und dann stark eingeengt; es blieb ein stark gelbes Öl übrig, aus dem sich nach eintägigem Stehen braunrote Flocken ausschieden. Durch Zusatz von Petroleumäther löste sich das Öl, während die rotbraunen Flocken ungelöst blieben. Sie wurden mehrmals mit Petroleumäther gewaschen und lösten sich hierauf in Chloroform mit gelber Farbe.

Einige Tropfen der Chloroformflösung wurden mit etwa 1 ml Alkohol und dann mit 0,5 ml einer alkoholischen Lösung von Zinkacetat + Spuren von Jod versetzt und dann mit einigen Tropfen Ammoniak versetzt. Im UV-Licht zeigte sich nun die für Bilirubin charakteristische rote Fluoreszenz. Empfindlichkeit: 1/12 000 000.

Als chemische Bestimmungs-Methode für Bilirubin z. B. im Blutserum und zur Unterscheidung von anderen Gallenfarbstoffen eignet sich die zuerst von EHRLICH angegebene Reaktion mit diazotierter Sulfanilsäure.[1]

In einer Zentrifugenröhre wird 1 ml Serum mit 1 ml Diazoreagens (1 g Sulfanilsäure diazotiert in 15 ml 25 %iger Salzäure auf 1 Liter verdünnt) gemischt und nach 2 Minuten mit Talk und 10 ml 0,05 n. Salzäure versetzt. Nach kräftigem Schütteln wird zentrifugiert, die klare Lösung, die farblos sein soll, wird abgegossen. Der Talk wird mit Wasser gewaschen, mit einer gemessenen Menge salzäurehaltigen Alkohols aufgerührt und zentrifugiert. Die nun blaue Lösung wird mit Pyridin versetzt (1 Tropfen je ml Alkohollösung), wodurch die blaue Lösung in rot umschlägt. Ihre Extinktion wird gemessen und mit einer Standardlösung verglichen.

Papierchromatographische Analysen der Gallenfarbstoffe in den Systemen 2,4 Lutidin–Wasser, Methanol–Wasser und Aceton–Wasser unter Zusatz von Ammoniak haben W. STICH und Mitarb.[3] ausgeführt.

Zur Untersuchung der aus dem Blutserum stammenden Stoffe der Ascitesflüssigkeit wurde auch der folgende papierchromatographische Versuch angestellt:

Munktell-Papier OB.	Flüssigkeit: 4 Tle n-Butanol, 1 Tl Eisessig, 1 Tl Wasser.
Entwicklung durch:	a) Ninhydrinlösung (Nin),
	b) Diazotierte Sulfanilsäurelös. (DSS).

Ascites-Flüssigkeit der Ratte 31. 2,5 ml Ascites gab mit 7,5 ml 96 %igem Alkohol eine Fällung (in welche die Substanz eingeht, die mit DSS Rotfärbung erzeugt). Die Fällung wird abzentrifugiert und mit etwas Alkohol gewaschen. Nach Eindunsten auf 2 ml wurde nochmals centrifugiert und die klare Lösung chromatographiert. Wir liessen diese 2 Flecken gleichzeitig wandern und entwickelten den einen Streifen mit Nin, den anderen mit DSS.

Das Chromatogramm zeigte etwa 14 freie Aminosäuren[4] im Alkoholextrakt; es fehlten anscheinend Aminosäuren mit R_f zwischen 0,35 und 0,75, unter diesen

---

[1] EHRLICH, Zbt. f. klin. Med. *4*, 721 (1883). — HIJMANS V. D. BERGH, u. MULLER, Biochem. Z. *77*, 90 (1916).

[2] W. J. H. BUNGENBERG DE JONG, Klin. Wschr. 1942, 885.

[3] W. STICH, R. KEHL und H. R. WALTER, Z. physiol. Chem. 289, 6 (1951); 178 (1953). — R. KEHL und W. STICH, S. physiol. Chem. *290*, 151 (1952).

[4] Über den Proteingehalt des Exudates in den Yoshida-Ascites-Sarkom-Ratten vgl. EULER und HASSELQUIST, Dieses Arkiv 7, 29 (1954).

Alanin und Methionin, vielleicht auch Phenylalanin. Dagegen sind anwesend Cystin und Cystein.

Der mit DSS entwickelte Streifen enthielt 2 gelbe Flecken unbekannter Substanzen, mit $R_f = 0{,}12$ und $0{,}29$, und einen dritten schwächeren Flecken mit $R_f = 0$.

Da man in Betracht gezogen hat, dass Bilirubin nicht aus Hämin, sondern direkt aus Hämoglobin umgelagert wird, so wäre zu vermuten, dass man im Blutserum, in der Galle usw. noch Verbindungen des Bilirubins mit solchen Proteinresten antrifft, welche am Hämoglobin haften. Nun kennt man zwar Molekularverbindungen des Bilirubins mit Glykokoll, Alanin[1] und Histidin, aber es ist uns nicht gelungen, stabile Aminosäure-Derivate des Bilirubins herzustellen.

Bekannt ist noch ein Dimethylester des Bilirubins (W. Küster). Über die Darstellung eines Produktes vom Schmpt. 211° mit Phenylhydrazin siehe S. 551.

Unbekannt sind Glukoside des Bilirubins. Sie interessierten uns deshalb, weil es möglich erschien, dass Einflüsse der Glukose auf Bilirubin im Ascites an eine chemische Bindung der Glukose geknüpft ist.

Injektion von Glukose in Ascites-Sarkom-Ratten.

Glukose beeinflusst den Stoffwechsel von Tumoren im allgemeinen, und so auch den Stoffwechsel der Yoshida-Ascites-Sarkome. Durch die Gegenwart von Glukose wird die Atmungsintensität der Sarkomzellen geändert. Gleichzeitig tritt eine Tumorhemmung durch die Glukose ein, wenn diese in höherer Koncentration als 15 mg/ml injiziert wird.

Wir haben den gelben Ascitesfarbstoff nicht nur auf seinen Gehalt an Bilirubin untersucht, sondern auch andere Gallenfarbstoffe wie Urobilin nach T. K. With[2] darin nachzuweisen versucht, ferner 2-kernige Derivate wie Pentdyopent nach Bingold[3]; dieselben begleiten in der Ratte das Bilirubin aber nur in sehr geringen Mengen. Ein noch unbekannter Begleiter des Bilirubins zeigte sich im Papierchromatogramm einer im Druck befindlichen Mitteilung.

An anderen Gallenbestandteilen konnten wir in der Leber nur kleine Mengen von Cholesterin nachweisen.

Versuchsratten, welche 7–9 Tage nach erfolgreicher Injektion von Tumor-Ascites[4] (ca. 1 Million Sarkomzellen) getötet wurden, enthielten im Blutserum (wie auch gleichzeitig im Ascites) sehr variable Mengen Bilirubin. Die folgenden Zahlen geben eine quantitative Vorstellung von dieser Variation.

Wir haben uns gleich der Frage zugewandt, auf welchen Umständen es beruht, dass in etwa 1/3 aller Fälle relativ grosse Bilirubin-Mengen in Ascites, Blutserum und dann in Fett, Haut usw. auftreten.

Es erschien auffallend, dass die Leber und Milz auch in solchen Fällen immer arm an Bilirubin waren, in denen sich viel Bilirubin in Blut und Ascites fand.

Das durch die injizierten Yoshida-Ascites-Sarkome erzeugte Exudat in der Bauchhöhle enthält oft noch rote Blutzellen.

---

[1] W. Küster, Z. physiol. Chem. *141*, 45 (1924).

[2] T. K. With, Z. physiol. Chem. *275*, 176 (1942).

[3] K. Bingold, Z. exp. Med. *99*, 325 (1936). — K. Bingold u. W. Stich, Med. Monatsschr. 1949, 243. — H. Fischer u. v. Dobeneck, Z. physiol. Chem. *263*, 135 (1940).

[4] Zur Definition von Ascites-Tumor siehe G. Klein, Experimental Cell Research *2*, 518 (1951).

Tabelle 1.

Ratte Nr.	γ Bilirubin per ml Serum	Ascites-Farbe
208	283	stark gelb
209	228	
211	0	farblos
219	0	farblos
225	0	farblos
226	420	sehr stark gelb

Die injizierten, im Exsudat an Zahl zunehmenden Sarkom-Zellen setzen sich infiltrierend an die Organe der Bauchhöhle an, und dürften die Fähigkeit besitzen, die Oberflächeschichten derselben zu perforieren, so dass die in der Leber befindlichen Gallenbestandteile weiter austreten können.

Den gelben Farbstoff im Ascites und im Serum haben wir immer beobachtet, wenn die Leber von Sarkomzellen, die zuweilen gelbe Flecken tragen, umgeben war. Auch wenn hartes Sarkom um den Hylus der Leber sitzt, zeigte sich oft reichliche Bilirubinbildung, und es lag nahe, dieselbe mit einer Störung im Gallenabfluss in Beziehung zu setzten.

Die meisten Tatsachen sprechen indessen nach unserer Auffassung dafür, dass es die Sarkomzellen sind, welche das Hämoglobin des Blutes in den Yoshida-Ascites-Sarkomratten zu Bilirubin abbauen. In der Regel ist nämlich das Bilirubin nicht nur im Ascites, sondern gleichzeitig im Blut angereichert.

Wie erwähnt, fanden wir in der Ascitesflüssigkeit neben Serumeiweiss auch viel Abbauprodukte desselben, entsprechend ihrer kapillaren Durchlässigkeit. Wir konnten experimentell nicht entscheiden, ob dieser Eiweiss- und Aminosäure-Gehalt von der Sauerstoffzufuhr abhängt. Eine Beziehung zwischen dem Bilirubingehalt des gelben Ascites und dem Gehalt des Blutes an Erythrozyten im gleichen Tier scheint nicht zu bestehen (siehe Ratten) Nr. 249 u. 250, S. 550).

Dass in einer so grossen Anzahl von unseren Ascites-Sarkom-Tieren Bilirubin so so reichlich vorkommt, reichlicher als in anderem Rattenmaterial erscheint, auffallend.

Ob die primäre Wirkung in einer serösen Entzündung in der Leber besteht, oder in unserem Rattenmaterial die Anordnung der Gallengänge den Ausfluss des Bilirubins in den Ascites erleichtert, können wir nicht entscheiden; wir halten letzteres für nicht wahrscheinlich.

J. P. GREENSTEIN[1] hat durch eingehende Versuche gezeigt, dass in Lebern von tumortragenden Ratten (zum Versuch kamen verschiedene Tumoren) der Katalase-Gehalt bzw. die Katalase-Wirksamkeit sehr wesentlich kleiner ist als in normalen Rattenlebern, eine Feststellung, die von EULER und L. HELLER durchaus bestätigt werden konnte.

Im Anschluss an letztgenannte Versuche haben wir[2] die einzelnen Leberlappen von normalen und von Buttergelb-Hepatome tragenden Ratten untersucht und einen analogen Unterschied im Katalase-Gehalt konstatiert. Auch in den Lebern von Yoshida-Ascites-Sarkom-Ratten ist dieser Effekt deutlich, wie nachstehende Versuchzahlen zeigen.

---

[1] J. P. GREENSTEIN u. Mitarb., J. Nat. Cancer Inst. 1–3 sowie 4, 283 (1944).

[2] EULER, H. HASSELQUIST, und E. ERIKSSON, Dieses Arkiv 4, nr 39 (1952).

Tabelle 2. Katalase-Wirkung.

Nr.	Ascites-Tumor-Ratten	k (Mittel)	kat. $f = k/g$ Leber
—	Tumorfreie Kontrolle	0,074	76
259	Ohne Zusatz	0,025	26
255	» »	0,016	18
264	+ Sbst. 167	0,033	34
270	+ » 166	0,027	31
273	+ » 166	0,029	33
248	+ » 167	0,068	72 (145)

Auf die Methodik der Messung und die Berechnung, die oft beschrieben wurde, brauchen wir hier nicht einzugehen.

Sbst. 167 = 6-Amino-chinaldin-4-carbons, siehe unten.

Sbst. 166 = 6-Amino-chinaldin-dicarbonsäure.[2]

Die k-Werte bzw. k_f-Werte stehen in guter Übereinstimmung mit den übrigen Beobachtungen an den Ascites-Sarkom-Ratten; beim Rückgang des Tumors steigt auch der Katalase-Wert wieder kräftig an (Ratte 248).

Nakahara isolierte mit Fukuoka 1949 eine thermostabile, nicht hitze-koagulierbare Sustanz, die in Wasser löslich ist und in Alkohol einen eiweissähnlichen Bestandteil aus menschlichem Krebsgewebe ausfällt, welche die Leber-Katalase wesentlich verkleinert. Sie wurde *Toxo-Hormon* genannt. Diese charakteristische Toxo-Substanz scheint die bekannte Katalasen-Verringerung in tumortragenden Ratten zu bewirken.

Nakahara konnte später zeigen, dass das Toxo-Hormon Eisen bindet[3] und so nicht allein mit der Synthese der Katalase sondern auch anderer eisenhaltiger biologischer Systeme in Konkurrenz tritt. Später fand Nakagawa die Katalase-Abnahme auch im Harn von Krebs-Patienten.

Tumor-hemmende Wirkung der 6-Amino-chinaldin-4-carbonsäure (Sbst. 167)

Wie schon mehrfach betont wurde eignet sich das Yoshida-Ascites-Sarkom besonders gut zu Untersuchungen für chemotherapeutische Zwecke, also zur Feststellung Tumor-hemmender bzw. -fördernder Substanzen. Wir haben an diesem Ascites-Sarkom früher den hemmenden Einfluss von Chinonen und Chinonmethiden und des Adrenochroms bestätigt und sind damit beschäftigt, das von A. Stoll dargestellte und beschriebene[1] Podophyllotoxin-glukosid zu untersuchen.

Ohne Wirkungen mit ähnlicher Methodik gefunden: Nikotinsäure und Nikotinsäureamid, Phloridzin und Cardiazol.

Unsere früheren Versuche mit dem Chinaldin-Derivat 167 waren an Jensen-Sarkomen durch Injektion ausgeführt worden und schienen zunächst cancerogen zu wirken.[2] Indessen gingen die Tumor-Ansätze nach 6–8 Wochen zurück, so dass wir nach den Erfahrungen mit dem Jensen-Sarkom, bei dem spontane Retrogradationen oft eintraten, nichts endgültiges über die Wirkung unseres Chinaldinderivates auszusagen wagten. Wir haben deswegen neue Versuche mit unserem Yoshida-Ascites-Sarkom angestellt, bei dem unter 250 Fällen nur vielleicht einmal ein spontaner Rückgang vorgekommen ist. Wir werden an anderer Stelle ausführlicher auf die Chinaldin-Versuche zurückkommen. Hier sei nur mitgeteilt, dass bei 7 Versuchen, bei welchen 3–6 mg 6-Aminochinaldin-4-carbonsäure zugleich mit der Ascites-Flüssigkeit eingespritzt wurden, in 5 Fällen eine *sichere Hemmung* der Tumorentwicklung eintrat, in 1 Fall war die Hemmung nicht so deutlich und in 1 Fall die Tumorentwicklung unbeeinflusst von der injizierten Substanz. Unten findet man Angaben über die Versuche an den Ratten 242, 243, 245, 246, 262 und 263.

---

[1] A. Stoll, J. Am. Chem. Soc. *76*, 1954, im Druck. Wir danken Herrn Kollegen A. Stoll bestens für die Überlassung der interessanten Substanz.

[2] Euler, H. Hasselquist und E. Eriksson, Dieses Arkiv 5, nr 23 (1952).

[3] W. Nakahara und F. Fukuska, Gann, 40, 45 (1949). — 42, 55 (1951). — 45, 67 (1954).

Die tumorhemmende Wirkung der Substanz 166 (der entsprechenden Chinaldindicarbonsäure) wurde sicher aber schwächer gefunden; vermutlich tritt im Rattenorganismus eine Decarboxylierung ein.

Beschreibung der Versuche

Ascites-Ratte 208. Graues Weibchen, Gewicht: 172–202 g, Inj. 14.4. 54. Getötet am 23.4. 54. Maximale Gelbfärbung, aber in gutem Zustand. Besonders stark gelb sind die unteren Hautschichten, die Bauchwandung und der Harn. Am Hilus der Leber lassen sich Krebsgewebe und Lebergewebe schwer unterscheiden. Reichlich feste, anhaftende Tumor-Zellen. Ascites-Flüssigkeit, etwa 4 ml, dick und stark gelb.

Bilirubin-Gehalt: 283 γ/ml Serum.

Das gleichzeitig getötete Kontroll-Tier (Normal-Ratte) war ein Weibchen, 300 g schwer, der Bilirubin-Gehalt des Blutes war nicht nachweisbar (kleiner als 10 γ/ml).

Ascites-Ratte 209, graues Weibchen, Gewicht: 149–165 g; Ascites-Flüssigkeit etwa 6 ml. Lebergewicht: 6,85 g; Milz 0,9 g. Bilirubin: 128 γ/ml Serum.

Ascites-Ratte 211, graues Weibchen (Rückkreuzung). Inj. 19.4. 54. Getötet am 24.4. 54 in gutem Gesundheitszustand. Im Blut kein Bilirubin nachweisbar, ganz wenig 1,5 ml, gelbgraue Ascites-Flüssigkeit; nur Darminhalt gelbgefärbt. Am Leber-Hilus deutlicher Tumor, schwächer aber deutlich im Mesenterium. Leber 11 g; Milz 1,5 g.

Ascites-Ratte 219. Nicht gelb.
0 γ Bilirubin/ml Serum.
0 γ Bilirubin/ml Ascites-Flüssigkeit.

Ascites-Ratte 225. Weibchen.
0 γ Bilirubin/ml Serum.
0 γ Bilirubin/ml Ascites-Flüssigkeit.

Ascites-Ratte 226. Weibchen
420 γ Bilirubin/ml Serum.
405 γ Bilirubin/ml Ascites-Flüssigkeit.

Versuche mit Sbst. 167 (6-Amino-chinaldin-4-carbonsäure)

Ascites-Ratte 243. Graues Weibchen der F_1-Generation, inj. 8.5. 54 mit 0,3 ml Ascites + 0,3 ml Lösung, 3 mg Sbst. 167 enthaltend.

Die Ascites-Flüssigkeit und die Versuchslösung wurden 30 Min. von der Injektion gemischt. Ratte in gutem Zustand, Haut nicht serös, nicht gelb.

Ascites-Flüssigkeit rötlich-grau, ohne Blutzellen, enthält 154 000 Ascites-Zellen/mm³ Ascites-Flüssigkeit.

Im Blut $7{,}5 \times 10^6$ Erythr./mm³ Blut.

Gewicht der Leber: 14,8 g; Gewicht der Ratte 345 g.

Ascites-Ratte 245 als Kontrolle, ohne Substanz 167. Gewicht der Leber: 14,7 g; Gewicht der Ratte: 395–437 g. Ascites-Flüss. enthält 178 000 Zellen/mm³. — Serum $7{,}0 \times 10^6$ Erythr.

Ascites-Ratte 246. Schwarzes Weibchen F_2, inj. mit Ascites 30 Min. nach dessen Entnahme. Seröse Schwellung der nicht-gelben Haut, Leber nicht angegriffen, nicht mit der Umgebung verwachsen, reichliche Tumor-Bildung.

Ascites-Ratte 242. Gelbes Männchen, Rückkreuzung. Inj. 0,3 ml Ascites + 3 mg Subst 167 in 0,3 ml Kochsalz-Lösung, injiziert am 8.5. 54, 30 Min. nach Mischung. Getötet: 15.5. 54. Guter Gesundheitszustand. Äusserlich nicht gelb, sehr wenig Ascites-Flüssigkeit, etwa 0,5–0,7 ml. Sehr wenig fester Tumor, sogar die Leber anscheinend tumorfrei. Fester hellgrauer Tumor 269 im Mesenterium. Lebergewicht 14,2 g. Milz 1,62 g. Gewicht der Ratte 319–365 g.

Ascites-Ratte 263, geb. 5.3. 54. Albino-Weibchen. Inj. 18.5. 54 mit 0,6 ml der Mischung von 0,3 ml Ascites-Flüssigkeit von 253 + 0,3 ml der Lösung von 3 mg Sbst. 167, die 21 St. im Kühlschrank gestanden hatte und vor der Injektion auf etwa 35° erwärmt wurde. Nicht gelb; mässiger Gesundheitszustand.

Blutzellen-Zählung: $7{,}0 \times 10^6$ Erythr./mm³ Blut.

Ascites-Ratte 262, wie Ratte 263; ziemlich schlechter Zustand; gelb. Blutzellen-Zählung: $6{,}3 \times 10^6$ Erythr./mm³ Blut.

Ascites-Ratte 249. $8{,}0 \times 10^6$ Erythr./mm³ Blut.

Bilirubin: Ascites-Flüssigk. 186 γ/ml. — Serum 249 γ/ml.

Ascites-Ratte 250. $9{,}7 \times 10^6$ Erythr./mm³ Blut.

Bilirubin: Ascites-Flüssigk. 186 γ/ml. — Serum 267 γ/ml.

Umsetzung des Bilirubins mit Phenylhydrazin in Chloroform

80 mg Bilirubin wurden in 50 ml Chloroform gelöst und mit einer Chloroform-Lösung von freiem Phenylhydrazin, entsprechend 200 mg Phenylhydrazinchlorhydrat versetzt. Aus der Reaktionsmischung fiel nach 16-stündigem Stehen im Dunkeln ein Reaktionsprodukt in Form kleiner gelber Nadeln aus, die nach Abfiltrieren und Waschen mit Chloroform bei 221° unterer starker Rotfärbung und Zersetzung schmolzen.

Die Substanz ist äusserst leichtlöslich in Methanol (kolloidale Lösung?), schwer löslich in Benzol, Chloroform, Äthylacetat und Äther. Sie gibt keine Reaktion auf Bilirubin.

Herrn Ingenieur Erik Eriksson danken wir für die Ausführung der Zählung der roten Blutzellen, der Ascites-Sarkomzellen und für die Katalase-Bestimmungen.

Stockholm, Universität, Vitamin-Institut und Institut für organ.-chemische Forschung.

Tryckt den 30 november 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 60

Read 22 September 1954

On the action of highly purified preparations of secretin and of pancreozymin

By Erik Jorpes and Victor Mutt

With 4 figures in the text

The dual mechanism of the external pancreatic secretion was discovered and studied by Pavloff and his pupils back in the previous century. They observed that the introduction of dilute hydrochloric acid into the duodenum of a dog with a pancreatic fistula gave rise to an intense secretion of a very dilute alkaline juice poor in enzymes and differing from the concentrated juice with a high enzymatic activity which followed an electric stimulation of the vagus nerve. It was in direct continuation of Pavloff's studies that Bayliss and Starling discovered secretin in 1902. The name hormone was coined in connection with this discovery, it being used for the first time in Bayliss and Starling's Cronian Lecture of 1904 (1).

It was quite natural that Pavloff should look upon secretin as the normal stimulant for the secretion of water and bicarbonate. Likewise Mellanby (2), who in 1925 elaborated a technique of purifying secretin through adsorption on bile salts, came to the conclusion that the secretin stimulates the secretion of water and bicarbonate exclusively. Harper and Vass (3), who in 1941 used a secretin purified by Mellanby's technique expressed the same opinion.

As to the stimulation of the enzyme secretion Buchstab (4) showed already in 1904 in Pavloff's laboratory that fat in the duodenum would stimulate the pancreas to secrete a juice rich in enzymes even if the vagus and the splanchnic nerves going to the gland were sectioned. In 1926 Farrell and Ivy (5) obtained in dogs in connection with meals a fairly normal enzyme secretion from a subcutaneous transplant of the tail of pancreas, indicating a humoral mechanism of stimulation. A similar secretory activity with the production of enzymes has recently been observed by Wang and Grossman (6, 1951) in a transplanted pancreas, with the ordinary nervous connections cut, when hydrochloric acid, amino acids or peptone were introduced into the duodenum of the dog. The existence of another humoral secretory mechanism, distinct from that of secretin, had thus very early been indicated. The existence of such a mechanism was definitely proved by Harper and Raper (7) in 1943 when they discovered pancreozymin.

It has been difficult to decide to what extent the two intestinal hormones, secretin and pancreozymin, mutually participate in the stimulation of the pancreatic secretion: Two factors cooperate in making the picture unclear—the occurrence of pancreozymin in less pure secretin preparations, and the influence of the nervous system on the pancreatic gland.

Prior to the discovery of pancreozymin HAMMARSTEN, ÅGREN and LAGERLÖF (8) in 1937, after injecting 15 cat units per kg body weight of a secretin with 20 or 80 cat units per mg in man, came to the conclusion that secretin stimulates the pancreas to "a real time rate of enzyme production" and that it is a physiological stimulator of the digestive pancreatic juice. In 1941 BARRINGTON (9) also found in Babkin's laboratory that secretin has a stimulating effect on the excretion of enzymes. He used in most of his experiments a secretin SI of IVY, KLOSTER, DREWYER and LUETH (10) and preparations purified by means of picrolonic acid with the technique of HAMMARSTEN, ÅGREN and WILANDER (11) and of GREENGARD and IVY (12). After the discovery of pancreozymin, which accompanies the secretin in crude preparations, these observations could be interpreted in a different way. LAGERLÖF (13) himself later expressed a more cautious view, particularly in discussing the action of secretin in man, where the nervous mechanism cannot be eliminated.

GREENGARD (14) in his review of 1948 claimed that "even in the case of pure secretin, an enzyme-free secretion has never been recorded to be elicited", and in the 1950 edition of his monograph BABKIN (15) cited BARRINGTON's results without any comments.

Lately the original view of MELLANBY has been accepted by THOMAS (16) in 1952, who claims that secretin according to the best evidence available has no effect on enzyme secretion.

Since we had at our disposal highly purified secretin preparations free of pancreozymin (JORPES and MUTT, 17, 18) and likewise active pancreozymin preparations, we had an opportunity to study this question in a large number of cat experiments. Using the same preparations WERNER and MUTT (19) analysed the effect of the two hormones in man.

Since nervous stimulation contributes to the secretion of enzymes it will be difficult to decide to what extent the low enzyme secretion observed during the prolonged secretin stimulation is caused by the secretin or is elicited through nervous influences. This is particularly the case in man. PAVLOFF (20), who had the same difficulties in studying the gastric secretion of the dog, tried to get rid of the psychic influences by using sleeping animals. WERNER and MUTT applied the same principle in children.

Histological evidence clearly indicates that secretin does not participate in the stimulation of the enzyme secretion. Already in 1909 BABKIN, RUBASHKIN and SAVICH (21) showed that stimulation of the vagal nerve of the cat gave an extensive extrusion of secretory granules, whereas, in spite of a copious secretion induced by hydrochloric acid on the duodenal mucosa, the glandular cells remained full of granules. Recently HARPER and MACKAY (22) in 1948 repeated the experiment and showed that the zymogen granules in the pancreas were not influenced by secretin, but were expelled from the cells by pancreozymin. In 1949 the same thing was demonstrated by DAVIES, HARPER and MACKAY (23) to occur in surviving pancreas slices. Secretin stimulated respiration but did not promote secretion of zymogen granules.

Our experiments and those of WERNER and MUTT with a highly purified secretin indicated that once the influence of the nervous mechanism is reduced sufficiently in the anesthetized cats and in infants, sleeping on hypnotics, the secretin alone will not stimulate to enzyme secretion.

Experimental

The secretin preparation, which we used, assayed 1000 cat units per mg according to the method of WILANDER and ÅGREN (24), as modified by JORPES and MUTT (25).

We wish to point out here that *the biological assay of secretin* according to this method is not carried out as described by GREENGARD in his aforementioned review. GREENGARD claims that "a piece of rolled up filter paper is placed in the exposed and opened duodenum of a urethanized cat, on which mixed secretions are collected". In actual fact small wads of tissue paper are held with forceps opposite the openings of the pancreatic ducts in the duodenum, and the secretion from the pancreas is sucked up quantitatively by them, without coming in contact with the duodenal mucosa. When a wad is drenched with juice it is transferred to a beaker, and a new wad is taken. At the end of the test period the wads are boiled with an excess of hydrochloric acid and the excess acid is titrated with sodium hydroxide. The cat unit is that amount of secretin which, if injected intravenously, will induce the pancreas to secrete alkali equivalent in amount to 0.1 ml 0.1 normal hydrochloric acid in a ten-minute period following the injection. The optimal amount of juice secreted for the assay is 1–1.5 ml, corresponding to 10–15 cat units. Of course this unit is only a provisional one, pending the introduction of an international secretin standard.

Determination of the pancreozymin activity. We applied the same technique for collecting the pancreatic juice following injections of pancreozymin into cats as used in the assay of secretin. We do so because collecting the pancreatic juice from the papilla Vateri does not damage the pancreas as a cannulation of the duct would do. For the same reason PAVLOFF (26) already in 1880 collected the pancreatic juice directly from the papilla in his long time fistula dogs. Furthermore the existence in the cat of an accessory duct makes it impossible to collect the total amount of pancreatic juice through a cannula in the main duct only.

A detail of some importance is that the bile duct has its opening in common with the pancreatic duct and the pancreatic juice may consequently become mixed with bile. This could be avoided by ligating the bile duct, which would, however, result in biliary stasis. It could also be cannulated, and the bile led off either into a container or, preferably, into the intestine, caudal to the field of operation on the duodenum. We have done this in a few instances only, since contamination of the pancreatic secretion with bile does not influence our results. The juice obtained in response to secretin was generally free of, or only slightly contaminated with, bile. Our pancreozymin preparations contained cholecystokinin, and the juice secreted in response to them was often strongly mixed with bile.

Since it is well-established that the lipase, amylase and tryptic activities in the pancreas run parallel, we have, for the sake of simplicity, analyzed only one of them —for technical reasons the tryptic activity.

Tryptic activity was determined after activation of the juice with enterokinase instead of with crystalline trypsin as used by *Doubilet* and *Troll* (27). The trypsin formed from trypsinogen by the enterokinase activates the chymotrypsinogen.

The secretion was collected in the ten-minute period following injection. It had a volume of 2–2.5 ml. The wads of paper imbibed with the juice were washed out with ice water, and the volume of the solution was made up to 25 ml. The pH of it was adjusted to 6.5–7.0 with acetic acid. For activation it was left standing for 12 hours at + 5° C with one mg of enterokinase, prepared according to KUNITZ (28), per ml solution. The tryptic activity in the activated solution was determined with ANSON's colorimetric method, as adapted to the assay of impure enzyme extracts by WERNER (29). In this method a denatured solution of hemoglobin is digested under standard conditions with the proteinase to be assayed, and the undigested protein is precipitated with trichloroacetic acid. The amount of split products in the filtrate is proportional to the proteinase activity. There are many methods for determining it. ANSON originally compared the colour given by the filtrate with Folin-Ciocalteus phenol reagent as compared with standard solutions of tyrosine. In this work we were interested only in the relative amounts of proteinase secreted in response to secretin and pancreozymin. For the sake of simplicity we have therefore only determined the optical density at 750 mμ of the colour produced by the phenol reagent, and used this density as a measure of the proteinase activity. We have throughout this work used a 10 mm cuvette.

The cats were anesthetized and operated upon as previously described by JORPES and MUTT (24). In some cases Nembutal was used for the anesthesia instead of chloralose. The secretin and pancreozymin were always injected intravenously. When studying the secretion of enzymes a continuous flow of pancreatic juice was maintained by the injection once every twenty minutes of 25 cat units of secretin per animal. At intervals pancreozymin was injected simultaneously with the secretin.

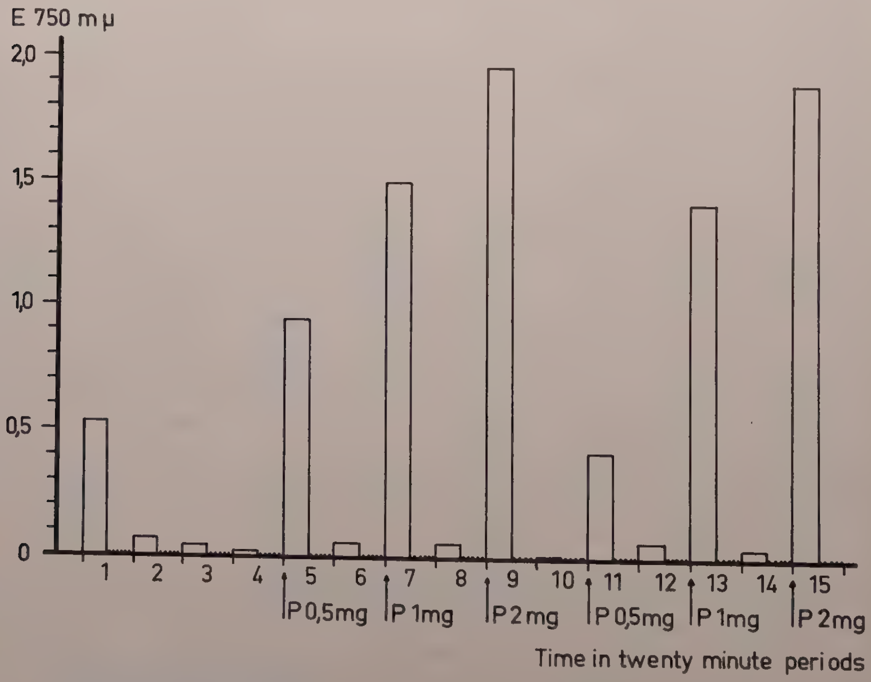


Fig. 1. Cat anesthetized with nembutal.—25 cat units of secretin were injected at the beginning of every 20-minute period. Pancreozymin was injected together with the secretin at *P*. Enzyme activity determined in the pancreatic juice in the ten-minute periods following injection of secretin or secretin together with pancreozymin.

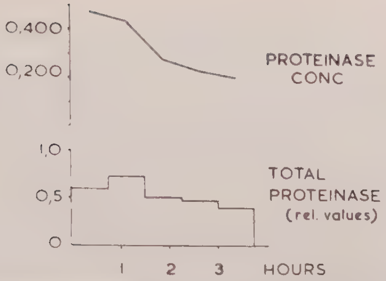


Fig. 2. Proteinase content of the duodenal juice during stimulation with secretin given every 15 min.—Infant 17 days old (diagn. Mongolism), during the test sleeping on phenobarbital.

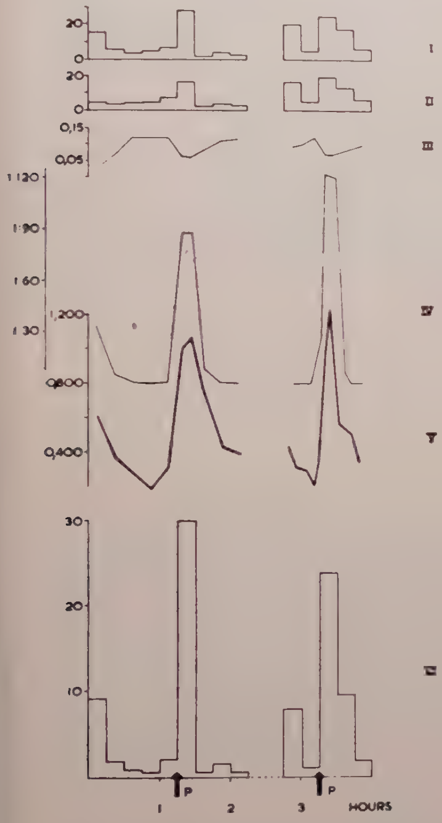


Fig. 3.

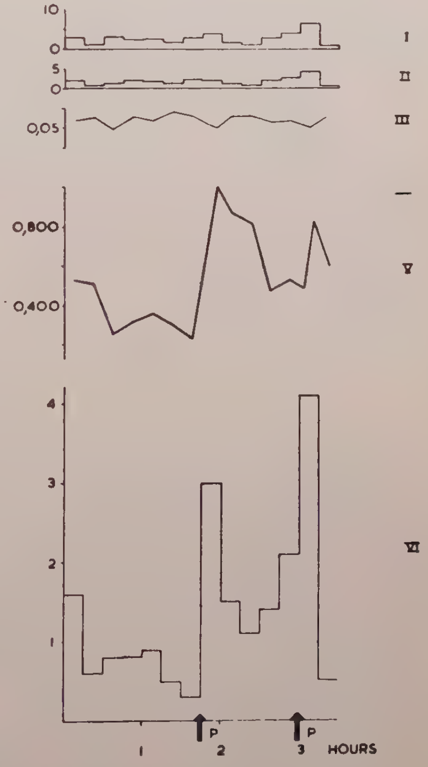


Fig. 4.

Fig. 3. Proteinase content of duodenal juice during stimulation with secretin (0.010 mg per kg injected every 15 min.) and with pancreozymin ($\frac{1}{2}$ mg per kg given at *P*).—Infant 1 year old (diagn. Hydrocephalus). *I*, Volume of duodenal juice, ml; *II*, Volume of obtained 0.1 N alkali, ml; *III*, Molarity of alkali; *IV*, Icterus index; *V*, Proteinase concentration of duod. juice; *VI*, Proteinase, total values.

Fig. 4. Proteinase content of duodenal juice during stimulation with secretin (0.010 mg per kg injected every 15 min.) and with pancreozymin ($\frac{1}{2}$ mg per kg given at *P*).—Infant 3 months old (diagn. Hydrocephalus). *I–VI:* see Fig. 3.

Results

In a series of one hundred cats we found that the enzyme content of the pancreatic juice dropped to a very low level during prolonged stimulation of the pancreas with secretin. This could not be due to fatigue of the gland since in every case injections of pancreozymin immediately increased the output of enzymes.

The output of enzymes in the ten-minute period following injection of pancreozymin, as compared with the previous secretin period was more than doubled in 99 of the hundred animals. In 66 animals the increase was more than tenfold and in 9 of these it was more than hundredfold.

The course of a typical experiment is evident from Fig. 1.

In three infants (Figs. 2, 3 and 4) sleeping on hypnotics WERNER and MUTT (19) repeatedly injected secretin at intervals of 15 minutes. This maintained a flow of pancreatic juice with a roughly constant volume per unit time. When the enzyme activity was determined in the juice it was found that starting with the first samples there was a continuous decrease in concentration of enzymes. The total output of enzymes likewise decreased.

In two of the cases (Figs. 3 and 4) the period of repeated stimulation with secretin was followed by an injection of pancreozymin simultaneously with the secretin. A new secretin period followed, whereupon pancreozymin was injected once more together with the secretin. In the fifteen-minute period following the injections of pancreozymin the concentration, as well as the total output of enzymes in the pancreatic juice, was increased many times as compared with the fifteen-minute period preceding this injection.

The low values for enzyme secretion during secretin stimulation were consequently not due to inability of the gland to secrete a juice with a higher enzyme concentration.

Summary

The opinion held already at a very early date by PAVLOFF and later accepted by such authorities as MELLANBY in 1925, HARPER in 1941, and THOMAS in 1952, that secretin stimulates the secretion of water and bicarbonate exclusively, has been confirmed in a series of experiments, in which a highly purified secretin, having an activity of 1000 cat units per milligram, was injected into a large number of cats. There is even in starving animals a tendency to a continuous production of enzymes with a slight accumulation in the pancreatic juice. Repeated injections of secretin cause a drop in the enzyme content of the juice to very low figures or some times to almost zero, from which level it can be raised by the tenfold or hundredfold through the injection of pancreozymin.

Similar results have recently been obtained in man.

Chemistry Department II, Karolinska Institutet, Stockholm, Sweden.

REFERENCES

1. BAYLISS, W. M., Principles of General Physiology Textbook. 1915 p. 706.
2. MELLANBY, J., J. Physiol. *60*, 85 (1925).
3. HARPER, A. A. and VASS, C. C. N., J. Physiol. *99*, 415 (1941).
4. Cited by BABKIN in his monograph of 1950.

5. Farrell, J. I. and Ivy, A. C., Am. J. Physiol. *78*, 325 (1926).
6. Wang, C. C. and Grossman, M. S., Am. J. Physiol. *164*, 527 (1951).
7. Harper, A. A. and Raper, H. S., J. Physiol. *102*, 115 (1943).
8. Hammarsten, E., Ågren, G., and Lagerlöf, H., Acta Med. Scand. *92*, 256 (1937).
9. Barrington, E. J. W., J. Physiol. *100*, 80 (1941/42).
10. Ivy, A. C., Kloster, G., Drewyer, C. E., and Lueth, H. C., Am. J. Physiol. *95*, 35 (1930)
11. Hammarsten, E., Ågren, G., Hammarsten, H., and Wilander, O., Biochem. Z. *264*, 275 (1933).
12. Greengard, H. and Ivy, A. C., Am. J. Physiol. *124*, 427 (1938).
13. Lagerlöf, H. O., Acta Med. Scand. Suppl. *128* (1942).
14. Greengard, H., Pincus and Thiemann, The Hormones p. 201 (1948).
15. Babkin, B. P., The Secretory Mechanism of the Digestive Glands. P. B. Hoeber, New York p. 54 (1950).
16. Thomas, J. E., Acta Gastroenterol. Belgica *15*, 811 (1952).
17. Jorpes, E. and Mutt, V., Arkiv f. Kemi *6*, 273 (1953).
18. Jorpes, E. and Mutt, V., Nature *172*, 124 (1953).
19. Werner, B. and Mutt, V., Scand. J. Lab. Clin. Invest. *6*, 228 (1954).
20. Pavloff, I. P., Die Arbeit d. Verdauungsdrüsen. Vorlesungen. Wiesbaden 1898 p. 95.
21. Babkin, B. P., Rubaschkin, W. J., and Savich, W. W., Arch. micr. Anat. *74*, 68 (1909).
22. Harper, A. A. and MacKay, I.F.S., J. Physiol. *107*, 89 (1948).
23. Davies, R. E., Harper, A. A., and MacKay, I. F. S., Am. J. Physiol. *157*, 278 (1949).
24. Wilander, O. and Ågren, G., Biochem. Z. *250*, 489 (1932).
25. Jorpes, E. and Mutt, V., Biochem. J. *52*, 328 (1952).
26. Pavloff, I. P., Sämtliche Werke II 1 p. 60; V p. 124. Akademie-Verlag Berlin, 1954.
27. Doubilet, H. and Troll, W., Gastroenterol. *19*, 326 (1951).
28. Kunitz, M., J. Gen. Physiol. *22*, 447 (1939).
29. Werner, B., Acta Pædiatr. *35*, Suppl. 6 (1948).

Tryckt den 31 december 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 61

Communicated 8 September 1954 by Arne Westgren

On ion exchange equilibria

IV. The silver-hydrogen equilibrium on Wofatit KS at low silver concentrations

By Erik Högfeldt

With 1 figure in the text

In the first paper in this series, Högfeldt et al. (1950) (in the following denoted by part I) the silver-hydrogen equilibrium was studied on Wofatit KS and Dowex 50. It was shown that the equilibrium quotient (see equation (2) below) was a variable and increased greatly with decreasing silver concentration. This behaviour required further studies, at lower silver concentrations than those which can be measured using emf-methods ($\sim 10^{-6}$ C), in order to find out if the equilibrium quotient attains a constant limiting value. Such a constant value should be expected in analogy with the results of Tompkins and Mayer (1947), who found constant values of the equilibrium quotient for a number of rare earth—ammonium systems on the tracer level using Dowex 50 resins.

To make measurements of silver concentrations lower than 10^{-6} C, radiometric methods with the radioactive silver isotope Ag^{110} were used. This isotope has a half-life of about 225 days, which makes it unnecessary to correct for the decrease of the activity with time during the measurements. It can be delivered with a specific activity of about 20 mCurie/g. In this investigation the silver delivered had an activity permitting the determination of concentrations down to $\sim 10^{-8}$ C and the concentration range could thus be increased by nearly two powers of ten.

This paper contains the results obtained with Wofatit KS. A following paper will be devoted to Dowex 50, where in addition the influence of the degree of cross-linking on the behaviour of the equilibrium quotient at low silver concentrations will be studied.

Experimental

Ion exchanger: The resin, Wofatit KS, was the same as that used and described in part I.

Chemicals and standard solutions: The chemicals and standard solutions were the same as those described in part I. The only difference was that the standard 0.1 C silver nitrate solution contained Ag^{110}, which was supplied from A.E.R.E., Harwell, Didcot, Berkshire, England. Solutions containing 10^{-2}—10^{-7} C Ag^{+} and of ionic strength 0.1 were prepared from the standard silver nitrate and nitric acid solutions.

Batches: The batches were made from the hydrogen saturated resin in the same manner as described in part I and shaken to equilibrium in all-glass pyrex bottles with sealed necks. The weights of the resin samples were adjusted to 1.0000 ± 0.0002 g.

All experiments were carried out in a thermostat room of 25.0 ± 0.2° C. The batches were equilibrated in a water thermostat placed in the thermostat room and kept at 25.0 ± 0.1° C or better.

Analyses: After equilibration, the resin and equilibrium solution were separated by sucking off the solution through a glass filter funnel and the resin was washed with about 100 ml dist. water. The hydrogen ions in the equilibrium solution were determined by pipetting 20.00 ± 0.03 ml and titrating with 0.1 C sodium hydroxide using methyl red-bromcresol green as indicator. The silver ions were determined by pipetting 10.00 ± 0.03 ml and transferring to a liquid β-counter (20th Century Electronics, type M 6) and the activity was measured. The concentration of silver in the solution was determined by comparison with the activity of a standard solution of known concentration.

For the determination of the silver content, the organic part of the resin was destroyed by wet combustion using a mixture of nitric and perchloric acids as described by Högfeldt and Kierkegaard (1954). The residue from the wet combustion was transferred to a 10 ml measuring flask, diluted to 10.00 ± 0.05 ml and the activity determined using the liquid β-counter. The hydrogen content of the resin was determined indirectly using a value of the saturation capacity (s_0) determined on separate samples by expelling the hydrogen ions with potassium nitrate and titration with standard sodium hydroxide. The saturation capacity was found to be 2.09_1 mmoles/g. Duplicates showed good agreement. This value was used for the calculation of the hydrogen content of the resin samples.

When calculating the concentration of hydrogen ions in the water solution it was assumed that the volume of this solution was 100 ml. On wetting the air-dried resin, water is taken up and the volume of the liquid solution will be less than 100 ml as pointed out by Samuelson (1944) as well as Lowen et al. (1951) and Bonner et al. (1952 a). In order to get an idea of how large this sorption of water may be, the following experiment was carried out. One gram samples of air-dried resin were shaken to equilibrium with 100 ml 0.100 C silver nitrate or nitric acid. After equilibration the aqueous solution was sucked off and its volume determined. For both cases a volume of 99 ml was obtained. As water adheres to the surface of the resin this value is a lower limit. This experiment shows that the amount of water sorbed by the resin is too small to make any corrections necessary.

Results

For the ion exchange reaction:

$$\mathrm{Ag^+ + HR \rightleftharpoons H^+ + AgR} \tag{1}$$

($R^- =$ anion framework of resin) we can define an equilibrium quotient ($\varkappa_{\mathrm{Ag,H}}$) as follows

$$\varkappa_{\mathrm{Ag,H}} = \frac{\{\mathrm{H^+}\}\,[\mathrm{AgR}]}{\{\mathrm{Ag^+}\}\,[\mathrm{HR}]} \tag{2}$$

where { } denotes activity and [ ] concentration.

In part I it was assumed that the quotient of the activity factors of nitric acid

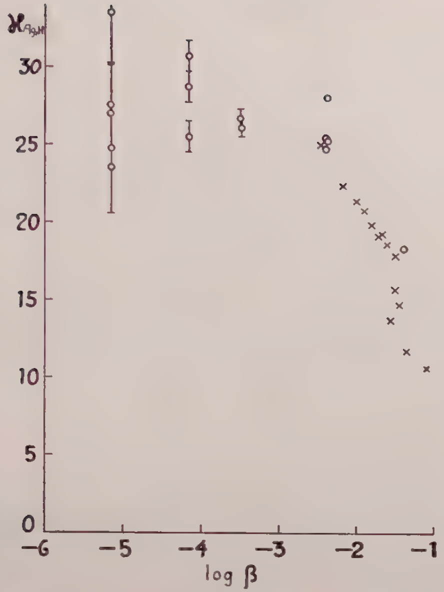


Fig. 1. $\varkappa_{Ag,H}$ is plotted as a function of log β where $\varkappa_{Ag,H}$ and β are defined by equations (3) and (4) respectively.

○ values obtained by radiometric methods.
× values obtained by emf-titrations.

and silver nitrate was constant throughout the concentration range studied and equal to unity. Equation (2) was then written in the form:

$$\varkappa_{Ag,H} = \frac{[H^+]\,[AgR]}{[Ag^+]\,[HR]} \tag{3}$$

This assumption was a reasonable one and later on a paper has been published by Bonner et al. (1952 b) dealing with that subject. They found at the ionic strength 0.1 C the quotient $f_{\pm HNO_3}/f_{\pm AgNO_3}$ was constant over a large concentration range and equal to 1.06. For low silver concentrations the ratio seemed to decrease, but no data are reported for such low silver concentrations as those used in this paper and for that reason $\varkappa_{Ag,H}$ defined by (3) will be used also in this investigation.

In Fig. 1, $\varkappa_{Ag,H}$ defined by (3) has been plotted against log β where the mole fraction of silver in the resin (β) is defined by:

$$\beta = \frac{[AgR]}{[AgR]+[HR]} = \frac{[AgR]}{s_0} \tag{4}$$

The open circles are values obtained using the radiometric method and the crosses values from the emf-titration method, Högfeldt (1952). The values in the overlap range of the two methods show a satisfactory agreement.

For the values obtained using the radiometric method the spread in $\varkappa_{Ag,H}$ arising from the statistical variation in the number of impulses counted is indicated as a vertical line. All measurements showing larger deviations than ±5 % in the silver balance were discarded.

From Fig. 1 we find that within the limits of experimental error $\varkappa_{\mathrm{Ag,H}}$ seems to attain a constant value ~ 26 within the interval $7 \cdot 10^{-6} \leq \beta \leq 4 \cdot 10^{-3}$. This result is in agreement with the results of TOMPKINS and MAYER (1947) who, as mentioned before, obtained constant values for the equilibrium quotient for a number of rare earth-ammonium systems on the tracer level using Dowex 50 resins.

Summary

The silver-hydrogen equilibrium is studied on Wofatit KS and ionic strenght 0.1 C at low silver concentrations using Ag^{110}. The equilibrium quotient seems to attain a constant value of ~ 26 within the interval $7 \cdot 10^{-6} \leq \beta \leq 4 \cdot 10^{-3}$ where β is the mole fraction of silver in the resin.

ACKNOWLEDGEMENTS

The author is indebted to Prof. L. G. SILLÉN and Mr. ERIK EKEDAHL for valuable discussions and helpful criticism. The Swedish Atomic Energy Committee is thanked for financial support. Thanks are also due to Mr. D. G. GILLAM for correcting the English text.

Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm, Sweden.

REFERENCES

BONNER, O. D., ARGERSINGER, Jr., W. J. and DAVIDSON, A. W. (1952 a), J. Am. Chem. Soc. *74*, 1044.

BONNER, O. D., DAVIDSON, A. W. and ARGERSINGER, Jr., W. J. (1952 b), J. Am. Chem. Soc. *74*, 1047.

HÖGFELDT, E., EKEDAHL, E. and SILLÉN, L. G. (1950), Acta Chem. Scand. *4*, 1471.

HÖGFELDT, E., (1952), Acta Chem. Scand. *6*, 610.

HÖGFELDT, E. and KIERKEGAARD, P. (1954), Acta Chem. Scand. *8*, 585.

LOWEN, W. K., STOENNER, R. W., ARGERSINGER, Jr., W. J., DAVIDSON, A. W. and HUME, D. N. (1951), J. Am. Chem. Soc. *73*, 2666.

SAMUELSON, O. (1944), Diss., Tekniska Högskolan, Stockholm.

TOMPKINS, E. R. and MAYER, S. W. (1947), J. Am. Chem. Soc. *69*, 2859.

Tryckt den 30 december 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 62

Mitgeteilt am 22. September 1954 durch ARNE WESTGREN und J. ARVID HEDVALL

Kontinuierliche Messung der Selbstdiffusion im Kalziumorthosilikat

Von ROLAND LINDNER und ERICH SPICAR

Mit 4 Figuren im Text

Das primäre Produkt bei der Reaktion zwischen Kalk und Kieselsäure ist das Orthosilikat (1), und daher war die Untersuchung der Atombeweglichkeit im Ca_2SiO_4 von besonderem Interesse innerhalb eines grösseren Arbeitsprogrammes zur Aufklärung des Mechanismus von Festkörperreaktionen (2).

Es wird in vorliegender Arbeit die Selbstdiffusion des Kalziums mittels des radioaktiven Indikators Ca-45 in Sintertabletten gemessen, während die Reaktion in Pulvermischungen, ebenfalls mit Ca-45 untersucht, in einer vorangegangenen Mitteilung behandelt wurde (3).

Methodisches

Herstellung der Probekörper

Nach neueren Erkenntnissen (4) existiert Ca_2SiO_4 in 4 Modifikationen: α-Ca_2SiO_4, beständig zwischen 2150 und 1456° C, α'-Ca_2SiO_4, beständig unterhalb 1456° C, das sich entweder bei 650° C in β-Ca_2SiO_4 oder bei 850° C in γ-Ca_2SiO_4 umwandelt.

Letztere Umwandlung geschieht unter Volumenvermehrung, was zum ,,Zerrieseln'' der Probekörper führt. Die Umwandlung soll nach TRÖMEL (5) durch wiederholtes Erhitzen von γ-Ca_2SiO_4 auf 1000° C und Wiederabkühlen zugunsten der α'-β Umwandlung unterdrückt werden, ein Befund, den wir indessen an *reinem* Material nicht reproduzieren konnten.

Zu haltbaren Sintertabletten gelangten wir nur, wenn wir das Silikat im Platinschiff auf nahezu 1700° C erhitzten und aus diesem Material Tabletten pressten. Hierbei wird indessen das Material erheblich mit Platin verunreinigt, und die damit erhaltenen Werte für die Diffusionskonstante haben nur orientierende Bedeutung. Sie werden weiter unten als Vorversuche näher besprochen.

Die Hauptversuche mit reinem Silikat wurden mit ,,instabilen'' Tabletten durchgeführt, die bei nur 800° C gesintert und (nach Belegung mit der dünnen radioaktiven Schicht) in der kontinuierlich arbeitenden Apparatur gemessen wurden.

Das Silikat war in jedem Fall durch Pulverreaktion zwischen analytisch reinem Kalziumkarbonat und Kieselsäure hergestellt worden, wobei zunächst gefällte Kieselsäure, später erheblich reinerer gepulverter Bergkristall verwendet wurde. Das Reaktionsgemisch wurde zwecks Erzielen guten Kontaktes zwischen den Körnern zu einer grossen Tablette zusammengepresst und eine Nacht im Platinschiff auf

1370° C erhitzt. Dies wurde nach Beseitigen äusserer, bräunlicher Verunreinigungsschichten wiederholt. Das fertige Produkt zeigte nur noch Orthosilikatlinien im Debye-Scherrer-Röntgendiagramm.

Messmethode

Die Messmethode basiert auf der Verringerung der gemessenen Radiokativität, die im Laufe des Versuches eintritt durch Diffusion im Festkörper mit folgender β-Strahlungsabsorption einer ursprünglich dünnen radioaktiven Schicht (6, 7, 8).

Diese Schicht wurde bei den unreinen Sintertabletten der Vorversuche durch Ausfällung radioaktiven Kalziumformiats (9) und Dekomposition zu Oxyd hergestellt, bei den reinen Tabletten der Hauptversuche durch Auftragen einer Suspension feinstgepulverten aktiven Silikates in einer 10 % Zaponlacklösung in Aceton (mit anschliessendem Trocknen und Verbrennen organischer Rückstände).

Bei den Vorversuchen konnten die Sintertabletten nach den verschiedenen Diffusionsabschnitten (Erhitzung in Silitstaböfen) bei Zimmertemperatur gemessen werden. Die Ermittlung der Diffusionskonstante erfolgte in bekannter Weise (6, 7, 8).

Bei dem reinen Material der Hauptversuche war die Messung bei Zimmertemperatur infolge des „Zerrieselns“ der Tabletten während der Abkühlung nicht möglich. Es musste eine besondere Versuchsanordnung aufgebaut werden, die das Messen der von der Oberfläche der Sintertablette ausgesandten radioaktiven Strahlung *während* der Erhitzung auf die Diffusionstemperatur gestattete und die im folgenden beschrieben sei.

Anordnung zur kontinuierlichen Messung

Die Aufgabe kann folgendermassen formuliert werden:

Es gilt, die weiche β-Strahlung einer Tablette von max. 1550° C Temperatur mittels eines Zählrohres, dessen Temperatur 30° C nicht übersteigen soll, mit gutem Wirkungsgrad zu messen.

Man muss also die Überführung von Wärme (durch Strahlung und Konvektion) möglichst einschränken, ohne die radioaktive Strahlung allzusehr zu schwächen. Anfänglich hielten wir einen Vakuumofen mit eingebautem Zählrohr für nötig; die entsprechende Konstruktion zeigte jedoch verschiedene Schwächen (u. a. mangelnde Temperaturkonstanz bei den allerhöchsten Temperaturen) und wurde zugunsten der im folgenden beschriebenen Konstruktion (vgl. Fig. 1) aufgegeben. Die Versuche wurden in Luft durchgeführt (der Ofen kann indessen evakuiert werden), die Wärme konnte aber ausreichend absorbiert und abgeleitet werden mittels der zwischen wassergekühlten Messingscheiben eingespannten 1–2 Duraluminiumfolien.

Die Schwierigkeit besteht darin, eine für Wärmestrahlung möglichst undurchsichtige und für die β-Strahlung durchlässige Folie zu finden, ausserdem natürlich noch möglichst kleinen Abstand zwischen Tablette und Zählrohr einzuhalten, um so einen grossen Öffnungswinkel für die radioaktive Strahlung zu gewährleisten. Ausserdem soll die Möglichkeit bestehen, die Anzeige des eingebauten Thermoelementes durch eine pyrometrische Messung der Tablettentemperatur zu kontrollieren. Fig. 1 zeigt, wie diese Aufgabe gelöst worden ist.

Der Ofen ist geometrisch und damit auch thermisch völlig symmetrisch gebaut. In der Mitte des Alundum-Rohres (A) — lichter Durchmesser 17 mm, Länge 60 mm, Wicklung für elektrische Beheizung 0,5 mm starker Platindraht — ist ein 1 mm hoher Ringwulst (B) aus Aluminiumoxyd-Zement aufgekittet. Auf diesem Wulst ruht ein dünnes Scheibchen aus Al_2O_3, das die Tablette

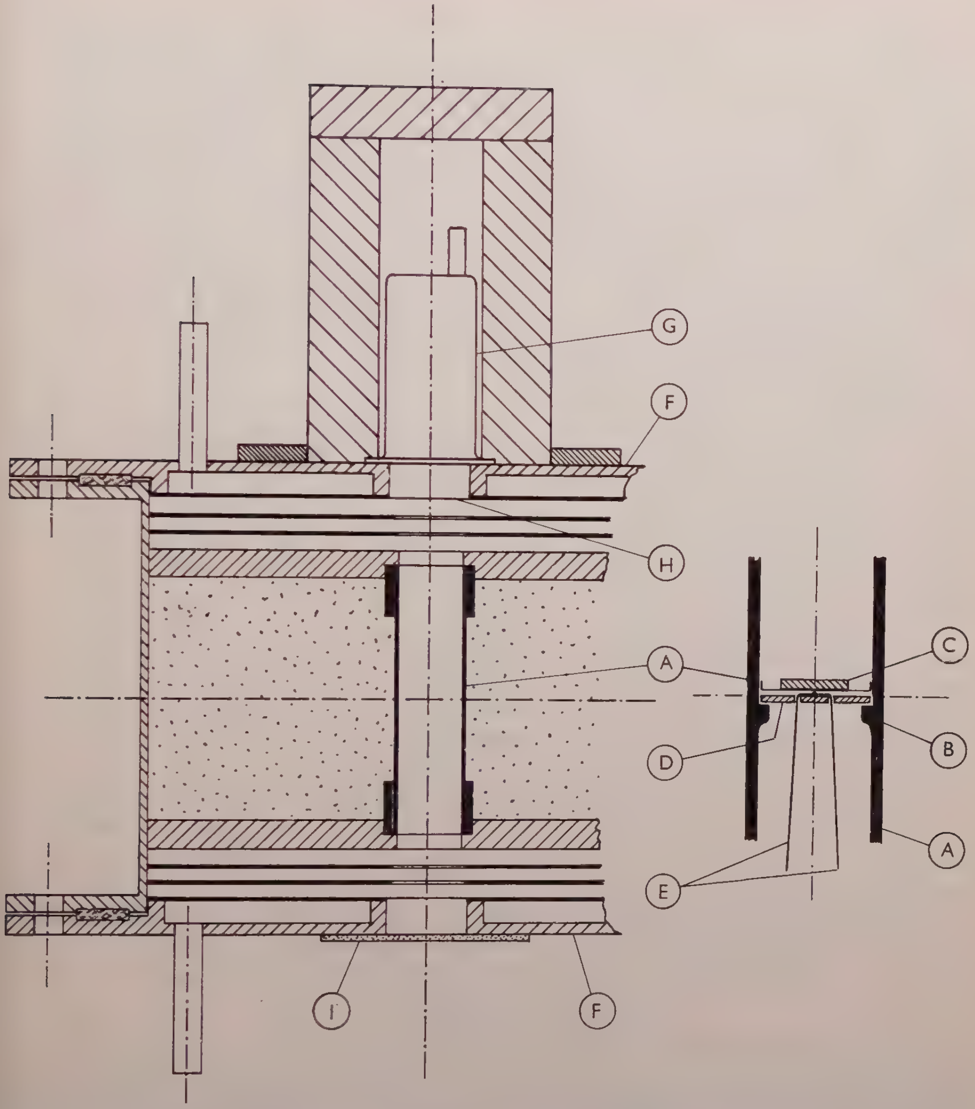


Fig. 1. Anordnung zur kontinuierlichen Messung von Selbstdiffusion in festen Stoffen bei hohen Temperaturen. (Die Lage des Präparates ist in der Teilzeichnung rechts im vergrösserten Maßstabe wiedergegeben.)

(*C*) trägt. Das Scheibchen (*D*) ist an zwei diametralen Stellen durchbohrt, und es ist ein Thermoelement, bestehend aus 0,2 mm starken Platin- bzw. Platin-Rhodiumdrähten (*E*), so durchgezogen, dass die Lötstelle genau unter der Tablette zu liegen kommt. Damit nicht das Kalziumorthosilikat der Tablette mit dem Aluminiumoxyd des Scheibchens reagiert, befindet sich zwischen Tablette und Scheibchen ein Platinblech. Der Abstand zwischen der Lötstelle des Thermoelementes und der Tablette beträgt so etwa $^1/_2$ mm und zwischen Tablettenoberseite und Unterseite des Al_2O_3-

Scheibchens 2 mm. Da die letztgenannten beiden Flächen symmetrisch und parallel zur thermischen Mittelebene des Ofens liegen, darf man annehmen, dass die auf beiden Flächen herrschenden Temperaturen die gleichen sind. Man kann also durch die untere Öffnung des Ofens das Scheibchen mit einem Pyrometer anvisieren und damit die Temperatur auf der abgewandten Oberfläche bestimmen. Diese Möglichkeit wird benützt, um das eingebaute Thermoelement auf die Genauigkeit seiner Anzeige zu kontrollieren. Durch die Verdampfung des in der Nähe der Thermoelement-Lötstelle befindlichen Platins wird die Konzentration in dieser verändert und damit bei längerem Betrieb auch die Anzeige des Thermoelementes gefälscht. Man kann dies jedoch korrigieren, wenn man bei neuem, kalibriertem Thermoelement die Temperatur auch gleichzeitig pyrometrisch misst und die beiden Werte in einem Diagramm gegeneinander aufträgt. Bei späteren Versuchen kann man durch einige wenige pyrometrische Messungen die Abweichung von der ursprünglichen Kurve feststellen und berücksichtigen.

Die beiden Stirnflächen (F) des Ofens sind doppelwandig ausgeführt. Zwischen den beiden Wänden zirkuliert Kühlwasser. Die Öffnung des Ofens zum Zählrohr (*G*) hin hat einen Durchmesser von 24 mm und ist mit einer 20 μ starken Duraluminiumfolie (*H*) verschlossen. Dieses Material erfüllt weitgehend die oben geäusserten Forderungen auf Durchlässigkeit für die β-Strahlung wegen seiner geringen Dichte und auf Undurchlässigkeit für die Wärmestrahlung wegen seines guten Wärmeleitvermögens. Ist die Ofenatmosphäre wasserdampffrei, so hält eine Folie einen Betrieb von 200 bis 300 Stunden aus. Der Abstand zwischen dem thermischen Mittelpunkt des Ofens, d.h. der Tablette, und der Folie beträgt nur 45 mm, zwischen Folie und Zählrohr 10 mm. Auf der Tablettenoberfläche wurden Temperaturen von 1500° C erreicht, dabei stieg die Temperatur im Zählrohr auf etwa 25 bis 30° C. Die Öffnung des Ofens zum Pyrometer hin (ebenfalls 24 mm Durchmesser) wird mit einer Blende mit kleinem Loch zugedeckt, ausserdem der Ofen an dieser Stelle mit einer aufgekitteten Quarzglasscheibe (*J*) verschlossen. Dadurch wird Zirkulation im Ofen vermieden, und man kann trotzdem das Innere mit einem Pyrometer anvisieren. Der Ofen ist so gebaut, dass er ohne grosse Schwierigkeiten auch im evakuierten Zustand betrieben werden kann, da alle Durchführungen in Pizein eingegossen sind.

Das Zählrohr befindet sich in einem starken, wassergekühlten Bleimantel. Der verwendete Impulsverstärker ist mit einem integrierenden Kreis versehen, so dass auf einem Messgerät die momentane Intensität des Präparates, ausgedrückt in Imp/min, abgelesen werden kann. Diese Anzeige kann man auch von einem schreibenden Milliamperemeter registrieren lassen und erhält so auf einem Papierstreifen (vgl. z.B. Fig. 3) eine Kurve, aus der man die unbekannte Diffusionskonstante bestimmen kann.

Berechnung der Diffusionskonstante

Die registrierten Kurven werden zur Auswertung graphisch gemittelt und die so erhaltenen Werte in einem Diagramm $A/A_0 = f(\sqrt{t})$ dargestellt. Ein Beispiel einer solchen resultierenden Kurve (durch Kreuze angedeutet) ist in Fig. 2 zusammen mit dem für die vorliegende experimentelle Anordnung speziell berechneten Verlauf der „Normalkurve" (für veränderlichen Absorptionskoeffizienten (s. weiter unten)) ausgezogen wiedergegeben. Es zeigt sich eine gute Übereinstimmung, wonach die dem berechneten Verlauf (Normalkurve) zugrunde liegenden Annahmen näherungsweise erfüllt sind. Die Streuung der Diffusionskonstante beträgt in diesem skizzierten Beispiel ± 10 %. Die noch vorhandenen Abweichungen werden weiter unten näher besprochen.

Um den jeweiligen Versuchsaufbau effektiv auszunutzen (und um die Temperatur der α–α'-Umwandlung genauer festzulegen), entschieden wir uns für die Durchführung sogenannter „Stufenversuche". Bei diesen verläuft der Versuch zunächst bei

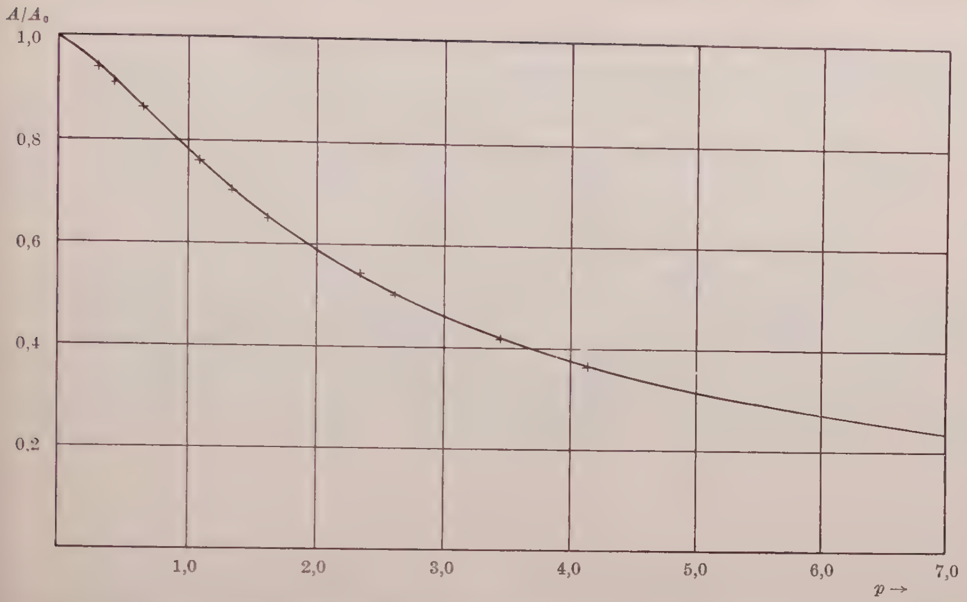


Fig. 2. Kurve zur Auswertung der Diffusionsversuche in vorliegender Anordnung (vgl. Text).

einer konstanten Temperatur, bis beispielsweise 80 % der Ausgangsaktivität erreicht sind (A-Versuch). Hierauf wird die Temperatur sprunghaft auf einen etwa 30° C höheren Wert gebracht und dort ebenfalls bei konstanter Temperatur belassen, bis die Aktivität auf etwa 60 % gesunken ist (B-Versuch), wonach durch nochmalige Temperaturerhöhung ein entsprechender C-Versuch angeschlossen werden kann. Auf diese Art erhält man in *einem* Versuchsgang Werte für die Diffusionskonstanten bei verschiedenen Temperaturen.

Theorie der „Stufenversuche"

Befindet sich zu Beginn des Versuches eine unendlich dünne Schicht der radioaktiven Substanz mit der Aktivität A_0 (pro cm^2) auf der Tablettenoberfläche, so wird die Konzentrationsverteilung dieser Aktivität beschrieben durch

$$c(x, t) = \frac{A_0}{\sqrt{\pi D_1 t}} e^{-\frac{x^2}{4 D_1 t}} .$$

Unterbricht man nach einer Gesamtzeit Δt_1 den Prozess und verändert die Diffusionskonstante sprunghaft, z. B. durch eine sehr rasche Temperaturänderung, auf einen neuen Wert D_2, so gilt für die weitere Ausbreitung obiger Konzentrationsverteilung

$$C(x, \tau) = \frac{1}{2\sqrt{\pi D_2 \tau}} \int\limits_0^\infty c(\xi, \Delta t_1) \cdot \left\{ e^{-\frac{(x-\xi)^2}{4 D_2 \tau}} + e^{-\frac{(x+\xi)^2}{4 D_2 \tau}} \right\} d\xi$$

$$= \frac{A_0}{2\sqrt{\pi D_2 \tau}\sqrt{\pi D_1 \Delta t_1}} \int\limits_0^\infty e^{-\frac{\xi^2}{4 D_1 \Delta t_1}} \left\{ e^{-\frac{(x-\xi)^2}{4 D_2 \tau}} + e^{-\frac{(x+\xi)^2}{4 D_2 \tau}} \right\} d\xi .$$

τ ist die Variable einer neuen Zeitzählung, die mit $t = \Delta t_1$ beginnt, und hängt mit der Gesamtzeit t des Prozesses so zusammen:

$$t = \Delta t_1 + \tau.$$

Durch Ausrechnung erhält man

$$C(x, \tau) = \frac{A_0}{\sqrt{\pi (D_1 \Delta t_1 + D_2 \tau)}} \cdot e^{-\frac{x^2}{4(D_1 \Delta t_1 + D_2 \tau)}}.$$

Die Funktionen $c(x, t)$ und $C(x, \tau)$ sind also ihrem Charakter nach gleich. Dies ist richtig, weil obiges $c(x, t)$ ein Fundamentalintegral der Fickschen Diffusionsgleichung ist; für jede andere Funktion $c(x, t)$ erhält man mathematisch unähnliche Funktionen $C(x, \tau)$ und kann daher nicht mehr mit Vorteil „Stufenversuche" ausführen. Solche sprunghaften Änderungen der Diffusionskonstante darf man beliebig oft nacheinander machen, der Ausdruck für die Konzentration $C(x, \tau)$ bleibt formal immer derselbe

$$C(x, \tau) = \frac{A_0}{\sqrt{\pi K}} e^{-\frac{x^2}{4K}}$$

mit

$$K = D_1 \Delta t_1 + D_2 \Delta t_2 + \cdots + D_{n-1} \Delta t_{n-1} + D_n \tau,$$

wobei τ mit der Gesamtzeit wie folgt zusammenhängt:

$$t = \sum_{j=1}^{n-1} \Delta t_j + \tau.$$

Mit dem Zählrohr wird die sogenannte Aktivität A der Tablette gemessen

$$A = \int_0^\infty C(x, \tau)\, e^{-f(x)}\, dx$$

(auf 1 cm² der Oberfläche bezogen).

In dem einfachen Falle, dass $f(x) = \mu x$ (μ ist die Absorptionskonstante für die radioaktive Strahlung in dem betreffenden Material) folgt der bekannte Ausdruck

$$A(\mu \sqrt{K}) = A_0\, e^{\mu^2 K} [1 - \Phi(\mu \sqrt{K})].$$

Die Bestimmung der einzelnen Diffusionskonstante $D_1 \cdots D_n$ geschieht folgendermassen:

Man bestimmt für den ersten Teil $0 < t < \Delta t_1$ der experimentell gemessenen Kurve $A/A_0 = f(\sqrt{t})$ durch Vergleich mit der Normalkurve

$$a(\mu \sqrt{Dt}) = e^{\mu^2 Dt} [1 - \Phi(\mu \sqrt{Dt})]$$

den Wert D_1. Mit diesem Wert sowie der bekannten Zeitdifferenz Δt_1 bestimmt man aus dem zweiten Teil der experimentellen Kurve D_2 usw.

Berücksichtigung des veränderlichen Absorptionskoeffizienten

Im allgemeinen ist der Absorptionskoeffizient für β-Strahlung keine Konstante, sondern von Energiespektrum, Geometrie der Messanordnung und Dicke der absorbierenden Schicht abhängig. In diesem Falle muss durch Absorptionsmessungen im kalten Ofen unter den sonstigen Bedingungen des Versuches die Funktion $f(x)$ bestimmt werden. Dies geschah in obiger Arbeit durch Verwendung dünner Aluminiumfolien bekannter Dicke als Absorber. Die experimentell gefundene Funktion $f(x)$ lässt sich mit guter Genauigkeit durch ein Potenzpolynom unabhängig von den Eigenschaften des verwendeten Absorbers darstellen durch

$$f(x) = M\varkappa(ax + b\varkappa x^2 + \cdots) \qquad 0 < x < \frac{1{,}8 \cdot 10^{-3}}{\varkappa}$$

$$f(x) = M(c + d\varkappa x + \cdots) \qquad \frac{1{,}8 \cdot 10^{-3}}{\varkappa} < x < \infty$$

$$x \text{ ausgedrückt in [cm]}$$

mit

$$\varkappa = \frac{\varrho_{\text{Absorber}}}{\varrho_{\text{Al}}}$$

$$M = \ln 10 = 2{,}3026.$$

In unserem Falle haben die Koeffizienten a, b, c, d den Wert:

$$a = 0{,}5789 \cdot 10^2 \quad c = -0{,}1000$$

$$b = 3{,}0864 \cdot 10^4 \quad d = -1{,}6900 \cdot 10^2.$$

Mit obiger Approximation für $f(x)$ lässt sich das Integral

$$A = \int_0^\infty C(x, \tau)\, e^{-f(x)}\, dx$$

geschlossen berechnen. Das Resultat als Funktion des Parameters $p = 10^3 \varkappa \sqrt{K}$ wird in Fig. 2 wiedergegeben und diente uns bei der Auswertung als Normalkurve. Im einfachsten Falle $n = 1$ (siehe oben) ist natürlich $K = D_1 t$.

Die Versuche und ihre Ergebnisse

Mit den eingangs erwähnten „stabilisierten", von uns als unrein angesprochenen Sintertabletten wurden schon 1949 (zusammen mit St. Austrümdal) Versuche zwischen 1165 und 1370° C nach der konventionellen Methode (9) (Erhitzung auf gewisse Zeitabschnitte, dazwischen Messung bei Zimmertemperatur) durchgeführt.

Die Ergebnisse lassen sich durch die untere gestrichelte Linie der Fig. 4 beschreiben. Da jedoch bezweifelt werden musste, ob sie für das reine, bei jenen Temperaturen stabile Orthosilikat charakteristisch sind, wurden die weiteren Versuche mit der kontinuierlich messenden Apparatur durchgeführt.

Hauptversuche nach der kontinuierlichen Methode

Die Versuche wurden so ausgeführt, dass der Ofen auf die gewünschte Temperatur gebracht und das thermische Gleichgewicht abgewartet wurde. Dann wurde mittels einer Pinzette eine Tablette eingelegt, der Ofen verschlossen und die Registrieranordnung in Gang gesetzt. Die Ofenspannung und damit auch die Temperatur wurde durch Eisen-wasserstoffwiderstände stabilisiert. Die Versuche erstreckten sich (je nach Temperatur) über Zeiten zwischen 2 und 80 Stunden. Nach dem Versuch wurde die Dichte der glühenden Tablette vor dem Zerfallen bestimmt, indem man die Tablette in einen kalibrierten Konus aus Pyrexglas fallen liess.

Die Aktivitätskurven auf den Registrierstreifen wurden wie erwähnt unter Verwendung des Abszissen-Masstabes $\sqrt{t}$ auf Millimeterpapier übertragen und rechnerisch mit der Normalkurve A/A_0 verglichen. Bei jedem Versuch wurde die Diffusionskonstante in 5 bis 10 Punkten der experimentellen Kurve bestimmt. Bei konstanter Versuchstemperatur sollten alle diese D.K. im Rahmen der Messungenauigkeit denselben Wert haben. Tatsächlich wurde jedoch ein Gang der D.K. beobachtet, wobei diese bei einigen Versuchen bis um einen Faktor von höchstens 1,5 zunahm.

Es ist zweckmässig, hier eine kurze Betrachtung der im untersuchten Fall vorliegenden Fehlerquellen einzuschalten.

Das Verhalten der Tablette während des Versuches weicht in mancher Hinsicht von den Anfangs- bzw. Randbedingungen ab, die man der Lösung der Diffusionsgleichung zugrunde legt. Dies führt auch zu dem erwähnten Gang der Diffusionskonstante mit der Zeit. Die in dem angegebenen Ausdruck für die Aktivität nicht enthaltenen Einflüsse seien nachstehend in der Reihenfolge ihrer Bedeutung genannt:

1) Das aktive Material sollte in einer unendlich dünnen Schicht auf die Tablette aufgebracht werden.

In Wirklichkeit war die aufgetragene Schicht einige 10–20 μ stark und bestand aus lose aneinander liegenden Kügelchen von aktivem Ca_2SiO_4.

Diese Teilchen mussten im Ofen erst miteinander und mit der Tablette zusammensintern und sich ausbreiten, bevor der Austausch von aktivem Material mit der unaktiven Tablette ungehindert stattfinden konnte. Folglich sank die Aktivität im Anfang langsamer als der berechneten Kurve entsprechend.

2) Da die Tabletten ziemlich porös waren ($\varrho \approx 1{,}8$ g/cm³; $\varrho_{\text{theor}} = 3{,}28$ g/cm³), als sie in den Ofen eingebracht wurden, erfolgte in der ersten Zeit eine ziemlich rasche Diffusion an den Korngrenzen und durch die Poren der Tablette hindurch. Die summarische Diffusionskonstante war im Anfang des Prozesses scheinbar grösser und wurde später kleiner.

3) Die Sinterung der Tabletten hat einen doppelten Einfluss: a) Die Dichte $\varrho(t)$ ist eine Funktion der Zeit und damit auch die Grösse $\varkappa = \dfrac{\varrho\ Ca_2SiO_4}{\varrho_{Al}}$. Wählt man bei der Auswertung das am Ende des Versuches gemessene ϱ, so ist dies zumindest für den allerersten Teil des Versuches falsch. — b) Die geometrischen Abmessungen (Durchmesser) der Tabletten werden kleiner (es können Änderungen des Durchmessers um 20 % vorkommen), und damit wird der für die Einstrahlung ins Zählrohr wichtige effektive Raumwinkel grösser, die Aktivität nimmt scheinbar zu, verglichen mit einer theoretischen Kurve.

Von den genannten Störeinflüssen war der erste der wichtigste, seine Beseitigung ist nur mit grossem Aufwand zu erreichen, z.B. durch Anwendung von sehr konzentriertem Ca-45, so dass die aufzutragenden Schichten genügend dünn gehalten werden können.

Die Genauigkeit der von uns erhaltenen gemittelten Diffusionskonstanten war für jeden einzelnen Fall ca.–20 % (die Reproduzierbarkeit war erheblich schlechter, was auf die unreproduzierbare Präparierung der Tabletten zurückzuführen ist).

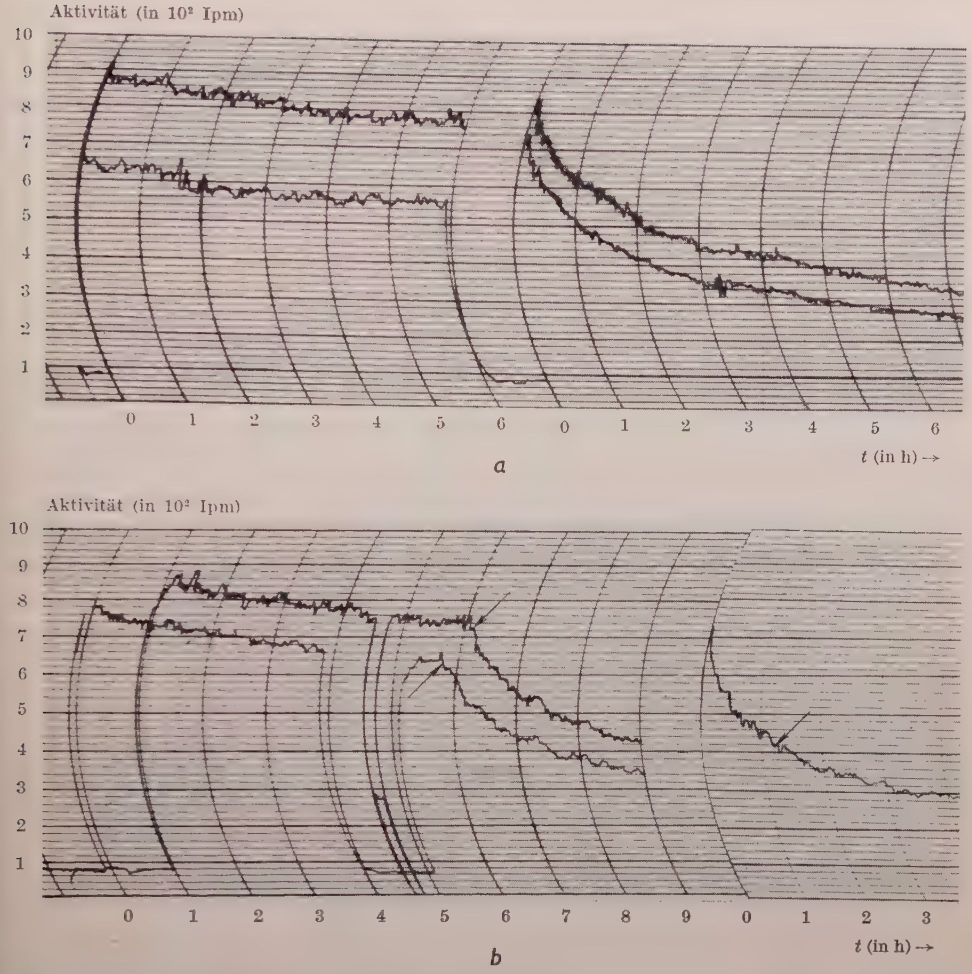


Fig. 3. Einige charakteristische Original-Registrierstreifen. — *3a:* getrennte Versuche bei tiefen bzw. hohen Temperaturen. *3b, links:* „Stufenversuch" innerhalb des Temperatur-Intervalles kristallographischer Umwandlung. *3b, rechts:* „Stufenversuch" ausserhalb des Umwandlungsintervalls.

Die Ergebnisse sind in der üblichen Darstellung $\log D = f(^1/_T)$ in Fig. 4 zusammengefasst. Es sind sowohl die an leicht verunreinigten Material (hergestellt aus Kalziumkarbonat und gefällter Kieselsäure) erhaltenen Werte (offene Kreise), wie auch die an reinem Silikat (hergestellt aus Kalziumkarbonat und reinstem Bergkristall) gewonnenen Werte (gefüllte Kreise) wiedergegeben.

Sie lassen sich bei tiefen bzw. hohen Temperaturen jeweils um (gestrichelte bzw. ausgezogene) Geraden gruppieren. Diesen entsprechen beim reinen Ortosilikat folgende Ausdrücke:

Unterhalb 1370° C: $D = 3{,}6 \cdot 10^{-2} \exp(-65000 \text{ cal/RT}) \text{ cm}^2 \text{ sec}^{-1}$,

oberhalb dieser Temperatur: $D = 2{,}0 \cdot 10^{-2} \exp(-55000 \text{ cal/RT}) \text{ cm}^2 \text{ sec}^{-1}$.

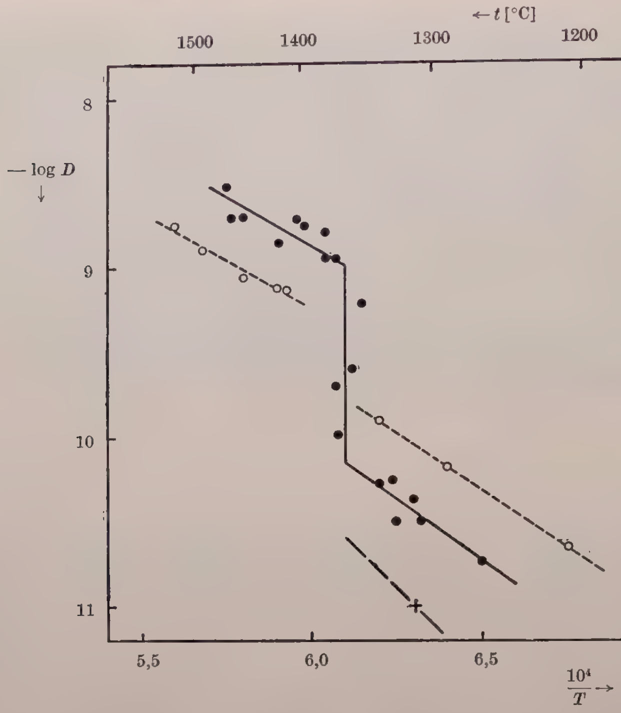


Fig. 4. Zusammenfassung der Ergebnisse in der Darstellung log $D = f(^1/_T)$. (Ausgezogene Kreise: Hauptversuche nach der kontinuierlichen Methode; offene Kreise: Vorversuche an verunreinigtem Material; die untere gestrichelte Linie zeigt den Verlauf bei den Vorversuchen an „stabilisierten“ Tabletten.)

Bei 1370° C liegt eine Unstetigkeit vor in Form einer sprunghaften Zunahme der Diffusionskonstante um den Faktor 15. Dies kann offenbar mit der α'–α Umwandlung des Ca_2SiO_4 in Zusammenhang gebracht werden, sodass wir den ersten der obigen Ausdrücke als für das α'–Ca_2SiO_4, den zweiten als für das α-Ca_2SiO_4 charakteristisch ansprechen wollen. (Diese Temperatur von 1370° C liegt erheblich tiefer als beim zuvorbetrachteten Literaturwerte der Umwandlung von 1456° C bzw. 1425° C. Die Umwandlung kann indessen stark durch Verunreinigungen beeinflusst werden.)

Zur Erläuterung der Messungen, insbesondere der im Umwandlungsintervall, seien die Registrierkurven der Fig. 3 noch einmal näher betrachtet. (Die zur oberen Kurve gehörende Temperatur ist stets zuerst angegeben.)

Fig. 3a zeigt in ihrem linken Teil 2 Versuche bei verhältnismässig tiefen Temperaturen (1315 bzw. 1290° C); im rechten Teil solche bei hohen Temperaturen (1450 bzw. 1514° C).

Dem erheblich steileren Abfall der Kurven entspricht eine über eine Zehnerpotenz grössere Diffusionskonstante. (Zwischen den Kurvenpaaren ist eine Zeitlang der Nulleffekt des Zählrohres von etwa 80 Ipm registriert.)

Fig. 3b dagegen zeigt links zwei „Stufenversuche“ bei denen zu einem bestimmten Zeitpunkt (durch Pfeil gekennzeichnet) die Temperatur von 1340 auf 1375 bzw. von 1330 auf 1385° C gesteigert wurde.

Man erkennt leicht, dass diese Temperatursteigerungen etwa die gleiche Wirkung haben wie die bedeutend grösseren der Fig. 3a, das heisst aber der Temperaturanstieg der Diffusionskonstante ist zum grossen Teil in einem kleinen Temperaturintervall zusammengedrängt, wie dies Fig. 4 quantitativ zeigte. Ausserhalb dieses Intervalles sind derartige Temperatursteigerungen nur von schwer erkennbarer Wirkung, wie in Fig. 3b rechts wiedergegeben, wo der Pfeil eine Temperaturänderung von 1420 auf 1462° C anzeigt.

Zusammenfassung

Die Selbstdiffusionskonstante von Kalzium in Kalziumorthosilikat-Sintertabletten wurde mit Hilfe des radioaktiven Isotops Ca-45 im Temperaturbereich 1165–1514° C bestimmt. Bei reinstem Material musste (wegen der Instabilität der Tabletten bei der Abkühlung) die radioaktive Messung *während* der Erhitzung auf die Diffusionstemperatur durchgeführt werden. Eine hierzu aufgebaute Anordnung, bestehend aus einem wassergekühlten Spezialofen, wassergekühltem Zählrohr und Registrieranordnung hat sich in ihrer Anwendung auf derartige Probleme bewährt.

Die Selbstdiffusionskonstante zeigt in ihrer Temperaturfunktion einen unstetigen Anstieg bei etwa 1370° C, der auf die Umwandlung $\alpha' \rightarrow \alpha\text{-Ca}_2\text{SiO}_4$ zurückgeführt wird. Die Temperaturfunktionen folgen den Ausdrücken

$$\alpha'\text{-Ca}_2\text{SiO}_4 : D = 3{,}6 \cdot 10^{-2} \exp(-65000 \text{ cal/RT}) \text{ cm}^2 \text{sec}^{-1}$$

$$\alpha\text{-Ca}_2\text{SiO}_4 : D = 2{,}0 \cdot 10^{-2} \exp(-55000 \text{ cal/RT}) \text{ cm}^2 \text{sec}^{-1}.$$

Wir danken *Statens Tekniska Forskningsråd* und *Chalmerska Forskningsfonden* für die Bereitstellung von Mitteln sowie Professor J. A. Hedvall für sein Interesse an der Arbeit.

Abteilung „Kristallkinetik", Institut für Silikatforschung, Chalmers Technische Hochschule, Göteborg (Schweden).

LITERATUR

1. Jander, W. und Hoffmann, E., Z. anorg. Chem. *218,* 211 (1934).
2. Lindner, R., z.B. J. Chem. Soc. London *1949,* 395.
3. Lindner, R. und Brune, U., Arkiv f. Kemi *5,* No. 26 (1953).
4. z.B. Trömel, G., Naturwissenschaften *36,* 88 (1949).
5. Trömel, G. und Möller, H., Fortschr. Mineralogie *28,* 80 (1949).
6. Zimen, K. E., Johansson, G. und Hillert, M., Journ. Chem. Soc. London *1949,* 392.
7. Lindner, R. und Johansson, G., Acta Chem. Scand. *4,* 307 (1950).
8. Hedvall, J. A., Einführung in die Festkörperchemie. Vieweg, Braunschweig 1952.
9. Lindner, R., Acta Chemica Scand. *6,* 468 (1952).

Tryckt den 30 december 1954

Uppsala 1954. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 63

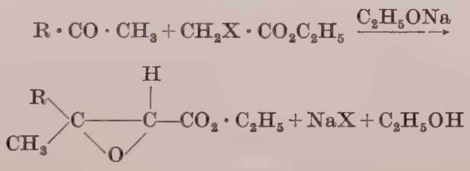
Communicated 27 October 1954 by ARNE FREDGA

The preparation of 2,4- and 3,4-dichlorohydratropic acids

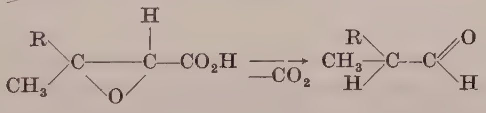
By GÖRAN ZDANSKY

In connection with work in progress at this institute on optically active plant growth regulators it was considered of interest to prepare 2,4- and 3,4-dichlorohydratropic acids.

Different methods have been tried for the preparation. The best method of synthesis seems to be that using glycidic esters prepared by DARZENS' condensation of the corresponding dichlorated acetophenones with ethyl chloroacetate, DARZENS (1), NEWMAN and MAGERLEIN (2), NERDEL and FRÖHLICH (1).



The glycidic ester obtained is hydrolyzed into free acid, which is decarboxylated and rearranged into the corresponding aldehyde:



Oxidation of the aldehyde obtained gives the corresponding acid.

The ketones have been obtained by oxidation of dichlorophenylmethylcarbinols. The carbinols have been prepared by Grignard synthesis from the corresponding dichlorobenzaldehydes.

Experimental

Dichlorophenylmethylcarbinols

In a 2 litre three-necked flask, provided with stirrer, dropping funnel and reflux condenser with calcium chloride tube, a Grignard reagent was prepared in the usual way from 16.8 g Mg-turnings and 100 g methyl iodide diluted with 200 ml absolute ether. The Grignard reagent was chilled with ice, and while stirring vigorously a solution of 120 g of dichlorobenzaldehyde (Eastman "Practical") in 750 ml absolute ether was added drop by drop. It is of importance that the stirring is highly efficient in order to avoid local excess of aldehyde, MARSHALL (4, 5, 6). Twenty minutes after the addition of the total amount of aldehyde the Grignard reagent was hydrolyzed by the addition of ice in small lumps. Dilute hydrochloric acid was then added until the basic Mg-salts were dissolved. The ether phase was separated and washed in turn with water, a solution of sodium hydrogen carbonate, a concentrated solution of sodium hydrogen sulphite and finally with water again. The solution was then dried overnight with anhydrous sodium sulphate. The ether was removed by distillation. The weight of the crude product, considered sufficiently pure for the continued experiments, was 128.5 g, representing a yield of 96 %.

The ketones were prepared by the following method indicated by LOCK and BÖCK (7):

3,4-Dichloroacetophenone

61 g 3,4-dichlorophenylmethylcarbinol were dissolved in 300 ml glacial acetic acid. 21.4 g CrO_3 were dissolved in 50 ml glacial acetic acid + 10 ml water, and added drop by drop to the solution of carbinol with vigorous stirring. The stirring was continued for 1 hour after the addition of the entire quantity of chromic acid. The reaction mixture was then poured into 1 litre of water and ice with simultaneous stirring, whereby the ketone separated. After a night in cool storage the product was filtered off and washed with cold water. The crude product was recrystallized from 75 % ethanol. The result was 44.3 g ketone with a melting point of 73–74.5° = a yield of 73 %. ROBERTS and TURNER (8) give a melting point of 76° for a product obtained by Friedel-Craft's synthesis with 40 % yield. LEONARD and BOYD (9) give the melting point as 72–75° and obtained a yield of 55.6 % by applying Sandmeyer's reaction on 3-amino-4-chloroacetophenon.

2,4-Dichloroacetophenone

85 g of 2,4-dichlorophenylmethylcarbinol were dissolved in 450 ml glacial acetic acid. To this solution was added dropwise a mixture of 26.8 g CrO_3 dissolved in 75 ml glacial acetic acid + 10 ml water under vigorous stirring. When all the chromic acid had been added, the stirring was continued for 3 hours, after which the reaction mixture was poured into water and ice. The oil which separated crystallised after some time and was filtered off. The mother liquor was extracted once with ether. The crude product obtained was of pasty consistency. For this reason it was oximated with hydroxylamine hydrochloride. The crude product was dissolved in 200 ml 80 % ethanol, and after addition of 31 g hydroxylamine hydrochloride and 24 g anhydrous sodiumcarbonate was refluxed for 1 hour. The reaction mixture was filtered in warm condition and left overnight to crystallize. Air-dried the oxime weighed 29 g and had a melting point of 146–147.5°. (LOCK and BÖCK (7) give the melting point of the oxime as 148°). The oxime was warmed during 1.5 hour with 150 ml concentrated hydrochloric acid + 150 ml water. After cooling the ketone was extracted with benzene. The benzene was distilled off and the ketone remained as an oil. Yield 26 g = 31.5 %

3,4-Dichlorohydratropaldehyde

The condensation of the ketone and the ethylchloroacetate with sodium ethoxide has to be carried out at low temperature, not exceeding + 5°. For every mole ketone 1.6 mole sodium ethoxide and 1.6 mole ester are to be used (2). It is possible either to add the condensing agent to the mixture of ketone and ester, as recommended by NEWMAN and MAGERLEIN (2), or to have the condensing agent dissolved in xylene, and to add a mixture of ester and ketone, which is more convenient.

In a 1 litre three-necked flask with stirrer, dropping funnel and reflux condenser with calcium chloride tube 7.4 g sodium were pulverized in 300 ml dry xylene. When the xylene had cooled, 19 ml absolute ethanol was added. (The ethanol was rendered absolute with magnesium ethoxide.) After about 1 hour the sodium powder was completely dissolved. After cooling in a bath of ice and salt until a thermometre in the xylene solution read −10° a mixture of 37.8 g 3,4-dichloroacetophenone, 38.5 g freshly distilled ethyl chloroacetate, and 50 ml dry xylene was added drop by drop with vigorous stirring. The rate of dropping was adjusted so that the temperature in the reaction mixture remained constantly between −10° and −5°. When all was added the chilling bath was removed and the stirring continued for 3 hours, until the reaction mixture had reached the temperature of the room. Finally the main part of the xylene was destilled off under reduced pressure. To the residue of the distillation was added 10 g sodium hydroxide dissolved in 100 ml water. The mixture was then refluxed with stirring during 3 hours. The mixture was acidified with dilute sulphuric acid. After heating to the boiling point the remaining xylene and the hydratropaldehyde were removed by steam distillation. 3.5 litres of the distillate were collected. About 7 g black tar remained in the flask. The distillate was extracted with ether. The extract was dried with sodium sulphate after which ether and xylene were distilled off. The aldehyde was distilled in vacuum through a short column. Boiling point 140–142°/12 mm Hg. 20.4 g aldehyde were obtained, corresponding to a yield of 50 %. The aldehyde is a colourless fluid with agreeable flower-like smell.

It appears probable that a better yield can be obtained if the time of reaction is prolonged, and the condensation is carried out in an inert athmosphere.

2,4-Dichlorohydratopaldehyde

The reaction was carried out exactly as for the 3,4-derivate. 5 g sodium were pulverized in 200 ml dry xylene and afterwards dissolved with 13 ml absolute ethanol. 26.0 g 2,4-dichloroacetophenone and 27.0 g ethyl chloroacetate dissolved in 25 ml dry xylene were added drop by drop. The result was 13.2 g aldehyde with a boiling point of 129–132°/11 mm Hg, representing a yield of 47.3 %. This aldehyde is also a colourless fluid, but with a somewhat less pronounced smell than the 3,4-dichlorohydratropaldehyde.

Oxidation of the hydratropaldehydes

The oxidation can be carried out with hydrogen peroxide in weakly alkaline solution. The acid obtained is of a high degree of purity, but the method gives low yields. Oxidation with silver oxide in alkaline solution is faster and gives better yields, DELÉPINE and BONNET (10).

3,4-Dichlorohydratropic acid

1.0 g 3,4-dichlorohydratropaldehyde and 1.7 g silver nitrate were dissolved in 10 ml ethanol + 2 ml water. 30 ml 0.5-C NaOH were added to this solution drop by drop with good stirring. After stirring during 18 hours the reaction mixture was filtered, and the reduced silver washed with warm water upon the filter. The collected filtrates were evaporated on a water bath to a volume of a few millilitres, which was then dissolved in water and the solution obtained extracted with petroleum ether in order to remove possible non-acid impurities. After cooling to 0° the solution was acidified with dilute sulphuric acid, when the hydratropic acid separated as an oil which soon crystallized. After being filtered off and dried the crude product weighed 0.90 g—a yield of 83 %. Melting point of the crude product: 40–43°. The acid was pure after five recrystallizations from petroleum ether. Melting point 42.5–43.5°.

0.09099 g substance: 7.751 ml 0.05349-C NaOH. — 0.01131 g substance: 5.346 ml 0.01943-C $AgNO_3$.

$C_9H_8O_2Cl_2$				
	Calc.	Equiv. wt.	219.06	Cl 32.37 %
	Found	» »	219.4	» 32.56 %.

2,4-Dichlorohydratropic acid

3.0 g 2,4-dichlorohydratropaldehyde + 5.1 g silver nitrate were dissolved in 20 ml ethanol + 7 ml water. Under vigorous stirring 90 ml 0.5-C NaOH were added to this solution drop by drop. 24 hours later the reduced silver was filtered off and washed with warm water upon the filter. The solution was evaporated, the residue dissolved in water, extracted once with petroleum ether and after cooling acidified with dilute sulphuric acid. The hydratropic acid separated as an oil which crystallized rapidly. The mother liquor was extracted three times with 10 ml portions of petroleum ether. The petroleum ether was evaporated and the residue crystallized immediately on cooling. The yield of the crude product was 2.6 g = 80 %. Melting point: 75–81°. Pure acid with a melting point of 82.5–83.5° was obtained after four recrystallizations from petroleum ether.

0.09026 g substance: 7.708 ml 0.05349-C NaOH. — 0.01352 g substance: 6.305 ml 0.01943-C $AgNO_3$.

$C_9H_8O_2Cl_2$				
	Calc.	Equiv. wt.	219.06	Cl 32.37 %
	Found	» »	218.9	» 32.13 %.

Some other methods of synthesis which have been tried

Some unsuccessful attempts have been made to prepare the dichlorohydratropic acids *over the corresponding nitrils.* For the preparation of the nitrils the ordinary method was tried in which a mixture of dichloro-α-phenethylchloride or -bromide with sodium or potassium cyanide in 50 % acetone-water was refluxed with vigorous stirring over periods as long as 48 hours. Subsequent hydrolysis yielded only traces of dichlorobenzoic acids, probably representing impurities in the dichloro-α-phenethylhalide. An attempt has also been made to use ethylene glycol as solvent as recommended by LEWIS and SUSI (11). The result was, however, a black tar-like reaction product which could not be hydrolyzed either with 40 % sulphuric acid or 20 % sodium hydroxide.

Direct chlorination of hydratropic acid with iodine or anhydrous ferric chloride as halogen carrier. The reaction was carried out with dry carbon tetrachloride as solvent. The chlorination of the hydratropic acid in this way is relatively easy, but difficulties arise in connection with the separation of the different reaction products.

Grignardation of dichloro-α-phenethylchloride and -bromide in the usual way results in complete "coupling", probably to the corresponding $\beta\gamma$-diphenylbutanes. Identical results with similar compounds are mentioned by OTT (12), STOBBE and POSNJAK (13), v. BRAUN, GRABOWSKI and KIRSCHBAUM (14). Trials were, therefore, made with the "Cyclic Reactor" described by ROWLANDS, GREENLEE and BOORD (15), and also by GAERTNER (16). This method also did not yield products with properties in agreement with those expected for the 2,4- and 3,4-dichlorohydratropic acids.

Summary

2,4- and 3,4-dichlorohydratropic acids have been prepared using the DARZENS glycidic ester synthesis. Attempts to prepare the acids using the corresponding nitrils, by direct chlorination of hydratropic acid or by the Grignard synthesis have not been successful.

Acknowledgements

The author whishes to express his thanks to Professor ARNE FREDGA for valuable discussions. The expenses involved in this investigation have been defrayed by a grant from the *Nobel Fund of the Royal Swedish Academy of Science* (*Kemiska prisgruppens särskilda fond*).

Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala, October 1954.

REFERENCES

1. DARZENS, G., Compt. rend. *139*, 1214 (1904).
2. NEWMAN, M., and MAGERLEIN, B., Org. Reactions *5*, 413. New York 1949.
3. NERDEL, F., and FRÖHLICH, H., Chem. Ber. *85*, 171 (1952).
4. MARSHALL, J., J. Chem. Soc. *105*, 527 (1914).
5. MARSHALL, J., J. Chem. Soc. *107*, 509 (1915).
6. MARSHALL, J., J. Chem. Soc. *127*, 2184 (1925).
7. LOCK, G., and BÖCK, E., Ber. *70*, 916 (1937).
8. ROBERTS, E., and TURNER, E. E., J. Chem. Soc. *129*, 1855 (1927).
9. LEONARD, N. J., and BOYD Jr., S. N., J. Org. Chem. *11*, 405 (1946).
10. DELÉPINE, M., and BONNET, P., Compt. rend. *149*, 39 (1909).
11. LEWIS, R. N., and SUSI, P. V., J. Am. Chem. Soc. *74*, 840 (1952).
12. OTT, E., Ber. *61*, 2139 (1928).
13. STOBBE, H., and POSNJAK, G., Ann. *371*, 301 (1909).
14. v. BRAUN, J., GRABOWSKI, A., and KIRSCHBAUM, G., Ber. *46*, 1280 (1913).
15. ROWLANDS, D. C., GREENLEE, K. W., and BOORD, C. E., Abstracts of Papers, American Chemical Society Meeting, Philadelphia, Penna., April 9 to 13, 1950 p. 8L.
16. GAERTNER, R., J. Am. Chem. Soc. *73*, 3934 (1951).

Tryckt den 18 januari 1955

Uppsala 1955. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 7 nr 64

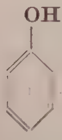
Vorgelegt am 22. April 1953

Einfluss der Azidität auf Phenole, Cyclodiene, Endiole und Anile

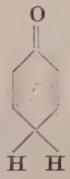
Von HANS VON EULER, H. HASSELQUIST und ARMIN GLASER

Mit 2 Figuren im Text

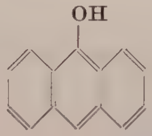
In neutraler, wässriger Lösung von Phenol ist die der Hydroxylform (I) entsprechende mesomere Ketoform (II) nur in ganz kleiner, noch nicht quantitativ festgestellter Menge anwesend. Immerhin erkennt man aus den Substitutionsreaktionen des Phenols, die von den an Benzol beobachteten so deutlich abweichen, dass Phenol in wässriger Lösung auch in der Ketoform reagiert. Eine Verschiebung des Gleichgewichtes I⇌II zur Ketoform durch Erhöhung der Azidität der Lösung konnte aber durch Messung der Extinktion im Gebiet 2500–3000 Å nicht nachgewiesen werden. Auch beim α-Naphtol ist eine solche Verschiebung gering; erst beim Anthranol (III) konnte K. H. MEYER[1], u. zw. durch Bromtitration einer 0,1 %igen Lösung, ein Keto-Enol-Gleichgewicht (11,5% Enol) feststellen. Die Extinktionsmessungen bei 2700 Å ergaben ein damit übereinstimmendes Resultat.[2]



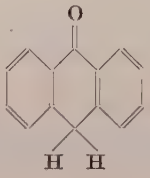
I



II



III



IV

Im Anthron (IV) ist die Methylengruppe derjenigen des Cyclopentadiens analog. Letztere reagiert nach THIELES[3] grundlegendem Befund mit Formaldehyd unter Bildung von Fulven, während Methylen-anthron aus Anthron und Formaldehyd nach einer Modifikation der Methoden von K. H. MEYER[4] bzw. von CLAR[5] erhalten wurde.

Sowohl das Fulven (es wurde stets mit dem stabileren Tetraphenylfulven gearbeitet) wie das Methylen-anthron reagieren an der Methylengruppe mit Nitrosodimethylanilin.

---

[1] K. H. MEYER, Liebigs Ann. Chem. 379, 55; 1911. — 396, 140; 1913. — Siehe auch JONES, J. Am. Chem. Soc. 67, 2127; 1945.

[2] O. CEDER, noch nicht veröffentlichte Versuche.

[3] J. THIELE, Liebigs Ann. Chem. 348, 4; 1906.

[4] K. H. MEYER, Liebigs Ann. Chem. 420, 135; 1920.

[5] E. CLAR, Ber. dtsch. chem. Ges. 69, 1686; 1936.

Die Komponenten in Pyridin gelöst und mit 1 ml Piperidin versetzt. Farbe anfangs grün dann braunrot und schliesslich rot-violett. Produkt aus Benzin, dann auch Alkohol umkrystallisiert, schwarze, glänzende Nadeln, Schmpt 141–142°. Mischschmelzpunkt mit der von KAUFLER und SUCHANNEK (Ber. 40, 525; 1907) aus Anthron und Nitrosodimethylanilin dargestellten Sbst. ergab 140–141°.

Vergleich der Kondensationsprodukte von Tetraphenyl-fulven und Tetraphenyl-cyclopentadien mit Nitroso-dimethylanilin

Das Dimethylaminophenylimin des 2,3,4,5-Tetraphenyl-fulvens wurde nach DILTHEY und HUCHTEMANN[1] dargestellt. Im Gegensatz zu diesen Autoren fanden wir den Schmpt bei 224–225°. Die Lösungsfarbe in konz. Schwefelsäure war olivgrün, eine tief-violette Farbe wurde nicht beobachtet.

Das Dimethylamino-anil des Tetraphenyl-cyclopentadienons wurde durch kondensation von Tetraphenyl-cyclopentadien mit Nitrosodimethylanilin unter den von DILTHEY u. HUCHTEMANN angegebenen Bedingungen dargestellt. Die Subst. stimmt mit dem von ZIEGLER und SCHNELL[2] unter anderen Bedingungen erhaltenen Produkt überein. Lösungsfarbe in konz. Schwefelsäure ist olivgrün. Schmpt 225–226°.

Nach ZIEGLER und SCHNELL[2] wird das Kondensationsprodukt von Tetraphenyl-cyclopentadien mit Nitrosodimethylanilin *durch Salzsäure* zu Tetraphenyl-pentadien gespalten.

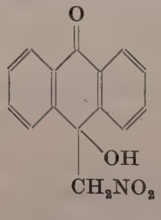
Das Kondensationsprodukt von Tetraphenyl-fulven mit Nitroso-dimethylanilin in Eisessig und Salzsäure wurde auf dem Wasserbad erhitzt; das Reaktionsprodukt wurde mit Wasser ausgefällt und abgesaugt. Nach dem Umkrystallisieren aus Benzol-Petroläther entstehen schwarze, glänzende Krystalle vom Schmpt 218°. Mischung mit 2,3,4,5-Tetraphenyl-cyclopentadien-(1)-on ergab keine Schmelzpunktsdepression.

Das Ergebnis ist gut vereinbar mit den von DILTHEY und HUCHTEMANN resp. von ZIEGLER und SCHNELL angegebenen Analysewerten.

ZIEGLERS Kondensationsprod. $C_{37}H_{30}N_2$.	Ber. N 5,58	Gef. N 5,70.
DILTHEYS Sbst. $C_{38}H_{30}N_2$.	Ber. N 5,46	Gef. N 5,53.

Einwirkung der Salpetersäure auf Methylen-anthron

Von besonderem Interesse erscheint das aus Methylen-Anthron und Salpetersäure entstehende Produkt



dessen Zusammensetzung $C_{15}H_{11}O_4N$ sich aus der folgenden Analyse ergibt.

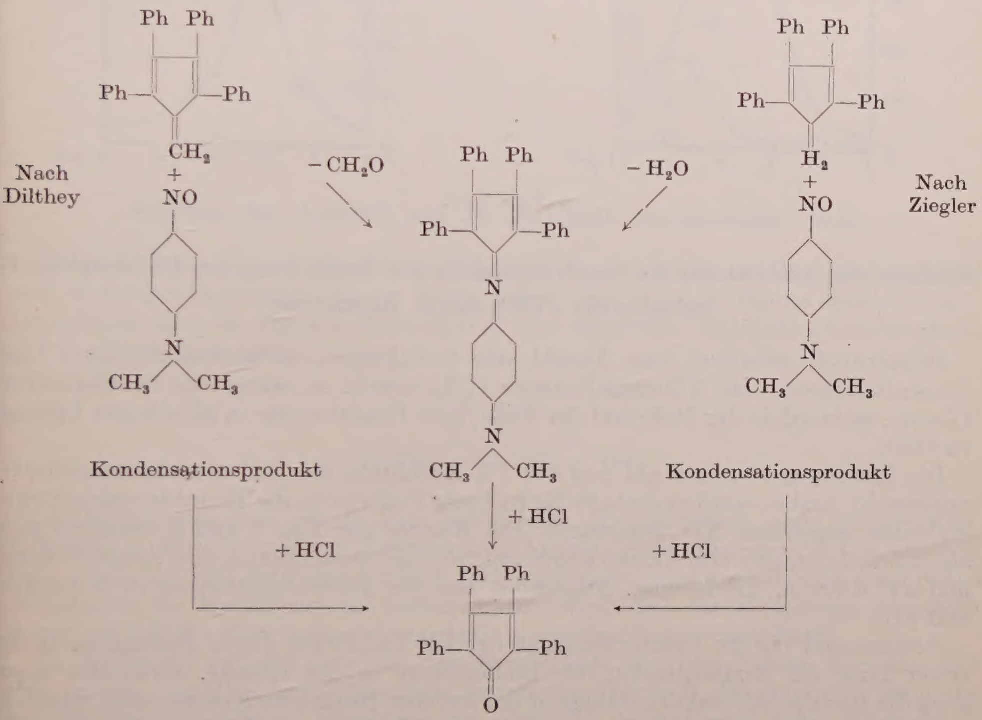
---

[1] W. DILTHEY u. P. HUCHTEMANN, J. pr. Chem. 154, 253; 1940.

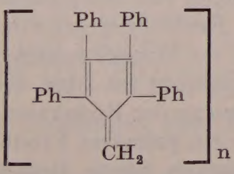
[2] K. ZIEGLER u. B. SCHNELL, Liebigs Ann. Chem. 445, 275; 1925.

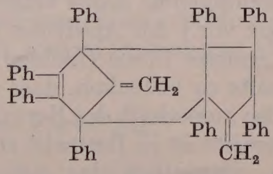
Ber. für $C_{15}H_{11}O_4N$	C 66,91%	H 4,12%	N 5,23
	gef.	67,69%	4,40%	5,13

Es spaltet sich unter Bildung von Antrachinon und (mit grosser Wahrscheinlichkeit) Nitromethan.


Nach Dilthey
Ph Ph
Ph
Ph
CH_2
+
NO
N
CH_3 CH_3
Kondensationsprodukt
$-CH_2O$
Ph Ph
Ph
Ph
N
N
CH_3 CH_3
+HCl
$-H_2O$
Nach Ziegler
Ph Ph
Ph
Ph
H_2
+
NO
N
CH_3 CH_3
Kondensationsprodukt
+HCl
+HCl
Ph Ph
Ph
Ph
O

Ein Polymerisationsprodukt des Tetraphenyl-Methylen-cyclopentadiens mit dem hohen Schmpt 370° konnte isoliert und analysenrein dargestellt werden (Sbst. 190).


Ph Ph
Ph
Ph
CH_2
n


Ph
Ph Ph
Ph
Ph
$=CH_2$
Ph
Ph
Ph
CH_2

Hypothetische Formel für n = 2

Analyse

Für $(C_{30}H_{22})_n$	ber.	C 94,20%	H 5,80%
	gef.	94,24%	5,44%

Über die Darstellung der hier erwähnten Substanzen siehe EULER u. H. HASSELQUIST, Chem. Ber. 86; 969, 1953.

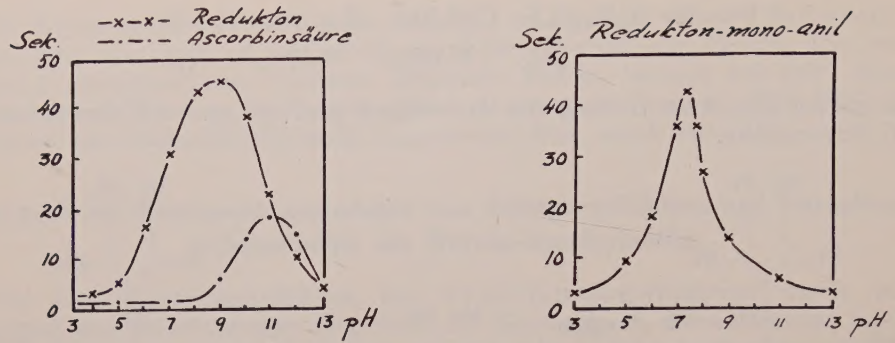


Fig. 1—2.

Einfluss der Azidität auf die Geschwindigkeit der Entfärbung des Dichlorphenol-indophenols (TR) durch Reduktone

Bekanntlich reduziert eine Anzahl von Reduktonen, u. a. Ascorbinsäure und Triose-Redukton, das Tillmans-Reagens (TR) sowohl in saurer wie in alkalischer Lösung, während in der Mehrzahl der Fälle diese Reaktion *nur* in alkalischer Lösung verläuft.

Die Beziehung zwischen pH und der TR-Reduktion war bisher nicht quantitativ untersucht, und es wurden deshalb für mehrere Reduktone die Reduktionsgeschwindigkeiten gegenüber TR festgestellt. Die Kurven der Fig. 1 und 2 beziehen sich auf 0,2 n, Lösungen von Triose-Redukton, Redukton-mono-anil und Ascorbinsäure und auf 0,005 n. TR-Lösung. Angegeben sind die Entfärbungszeiten in Sekunden und pH.

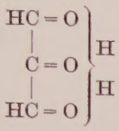
Bestimmend für die Geschwindigkeit der TR-Reduktion durch Reduktone ist in erster Linie die Konzentration der Endiol-Form in der Lösung. Zweifellos muss auch die spezifische Reaktionsfähigkeit der anderen Reaktions-Komponente, nämlich des Tillmans-Farbstoffes die Geschwindigkeit mitbestimmen. (In diesem Institut hat deswegen O. CEDER, das Spektrum des Dichlorphenol-indophenols im Gebiet 2000–4000 Å bei den Aziditäten pH = 2, 3,5, 5 und 11, aufgenommen. Für eine Reihe anderer Phenol-indophenole liegen eingehende Messungen von SCHWARZENBACH[2], MOHLER und SORGE vor.) Als Ausdruck für die totale Reaktionsgeschwindigkeit wurde deswegen an anderer Stelle (Liebigs Ann. Chem., im Druck) angegeben $c\,k \times c_1 k_1$. Nun ist einerseits zu bedenken, dass der Umschlagspunkt rot–blau des TR bei etwa pH 4,5 liegt, also ausserhalb des Bereiches des Minimums der Reduktionsgeschwindigkeiten. Andererseits ist in Betracht zu ziehen, dass ein primäres Produkt der beiden Komponenten, vermutlich das *Salz* der mittelstarken Säure Redukton und der Farbstoffbase die Reduktion vermittelt.

Was die reaktionsvermittelnde Struktur der beiden Reduktone betrifft, so wurde dieselbe früher mehrfach besprochen, zunächst in Zusammenhang mit der Tatsache, dass Redukton in alkalischer u. saurer Lösung leicht mit Semicarbazid und Hydrazin reagiert aber in neutraler Lösung stabil ist. Wir haben zunächst eine 4-Ring Formel in Betracht gezogen und bringen die vollkommene Symmetrie des Moleküls in Formel

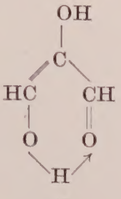
---

[1] EULER, H. HASSELQUIST u. O. CEDER, Liebigs Ann. Chem. *581,* 198 (1953).
[2] G. SCHWARZENBACH, H. MOHLER und J. SORGE, Helv. 21, 1636; 1938.

I zum Ausdruck, erwägen aber auch mit F. Arndt und B. Eistert das Auftreten einer Wasserstoffbrücke im Gebiet der Stabilität (Formel II).



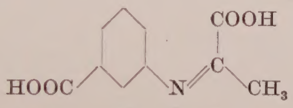
I



II

Einfluss der Azidität auf die Stabilität verschiedener Anile

An einer früher hier[1] dargestellten Substanz die sich leicht aus Brenztraubensäure und m-Amino-benzoesäure in der Kälte bildet, wurde von O. Ceder durch Extinktions-Messungen im Bereich 2300–3500 Å eine erhebliche Stabilität (Aziditätsgrade pH 3–pH 10) festgestellt.



Redukton-mono-anil wurde von Euler und Hasselquist[2] sowie von Cocker, O'Meara u. Mitarb.[3] und von Eistert und Hasselquist[4] näher besprochen. Das Na-Salz sowie das HCl-Salz sind kürzlich hier beschrieben und untersucht worden.[5] Aus Messungen der Extinktion an beiden Salzen ergab sich die auffallende Stabilität dieses als Schiff's Base betrachteten Anils gegenüber Säuren und Basen.

In Rücksicht auf die zwischen den Ergebnissen der irländischen Forscher und den unseren bestehenden Differenzen wurde von Herrn H. Hasselquist nun eine erneute Strukturprüfung unternommen, über die er in diesem Arkiv Bd 6, Nr 37 (1953) berichten wird.

Wir haben bis jetzt hauptsächlich die Stabilität der Produkte aus Anilin und Salicylaldehyd sowie aus p-Bromanilin und Zimtaldehyd und zwar durch Aufnahme der Spektren im UV untersucht. Die Ergebnisse werden im Anschluss an die Arbeiten von Gaouck und Le Fèvre (Soc. 1938, 741) sowie Curban und Chaput (J. Am. Chem. Soc. 69, 1134; 1947) besprochen.

Universität Stockholm, Inst. für organ.chem. Forschung.

---

[1] Euler, H. Hasselquist u. E. Eriksson, Dieses Arkiv 5, Nr 23; 1953.
[2] Euler, H. Hasselquist u. U. Lööv, Arkiv f. Kemi etc. 26 A, Nr 17; 1948.
[3] W. Cocker, R. A. Q. O'Meara, J. C. P. Schwarz u. E. R. Stuart, J. Chem. Soc. 1950, 2052.
[4] B. Eistert u. H. Hasselquist, Dieses Arkiv 4, Nr 12; 1952.
[5] Euler, H. Hasselquist u. O. Ceder, Liebigs Ann. Chem., *581*, 198 (1953).

Tryckt den 18 januari 1955

Uppsala 1955. Almqvist & Wiksells Boktryckeri AB